Vol. 36, No. 2 June 1949

THE ANNALS OF APPLIED BIOLOGY

EDITED FOR THE ASSOCIATION OF APPLIED BIOLOGISTS

BY

R. W. MARSH

AND

I. THOMAS

WITH THE ASSISTANCE OF

F. C. BAWDEN H. MARTIN
D. J. FINNEY D. J. WATSON

CAMBRIDGE UNIVERSITY PRESS
CAMBRIDGE, AND BENTLEY HOUSE, LONDON
AGENTS FOR U.S.A.
THE UNIVERSITY OF CHICAGO PRESS
CANADA AND INDIA: MACMILLAN

CONTENTS OF VOL. 36, NO. 2

STUDIES OF WIREWORM POPULATION

III. SOME EFFECTS OF CULTIVATION

BY GEORGE SALT, SC.D. (*King's College*)
AND F. S. J. HOLLICK, PH.D (*St John's College*)

From the Zoological Laboratory, University of Cambridge

(With 5 Text-figures)

The number of wireworms in successive collections from five Cambridgeshire fields declined during the first year of cultivation to 25 % of the number present before the fields were ploughed from old grass. The decline in numbers was accompanied by a change in the composition of the wireworm population; the marked inverse correlation between number and size of larvae, which held for the wireworm populations under grass, progressively altered towards uniform numbers of the different size-groups. The incidence of natural control on the different sizes of larvae was obscured by their growth, and more medium-sized and large larvae were lost than would appear from the population-size histograms.

Three groups of factors are considered as contributing to the decline in numbers after ploughing. Direct observation showed that many wireworms were destroyed in the actual process of cultivation. Evidence was insufficient to prove that the physical condition of cultivated soil adversely affects the number of wireworms. Comparison of the smaller size-groups in pasture and in the cultivated fields showed that the wireworm population failed to replenish itself under arable conditions.

INTRODUCTION

In what was perhaps the most significant contribution to the literature of the wireworm problem for at least a decade, Roebuck (1924) showed that the wireworm population of pastures declined rapidly after ploughing, and continued to fall steadily if the fields remained in cultivation. In four fields, Roebuck found an average wireworm population of 414,000 per acre the year after they were ploughed from grass; 326,000 the second year, 227,000 the third, and 129,000 the fourth. Five years' cultivation appeared to him to be sufficient to reduce the number of wireworms in most fields to a point at which any crop could be grown with safety. This observation has in general been amply confirmed by other workers. Indeed, the fact that wireworm populations decline after ploughing and subsequent cultivation has become a matter of common knowledge.

Two developments would seem to follow naturally and immediately upon Roebuck's observation: (1) a study of the factors that bring about the decline in wireworm numbers; and (2) an investigation to discover whether the decline can be accelerated, by different methods of cultivation, or by cultivation at particular

times of the year. The second of these developments requires the equipment of an agricultural institute or the powers of a government department, and is clearly beyond the range of two private and academic workers. There are aspects of the first, however, on which we have been able to collect some information. Although it is incomplete, we publish it here so that it may be of use to those who eventually tackle what could be made a very interesting ecological investigation.

Our information can be conveniently arranged under three headings:

(1) *Quantitative.* A number of pastures were sampled while still under grass, and at intervals of about 8 weeks for a few months after they were ploughed. The wireworms were collected from these samples by a method that is considered to extract all the wireworms of all stages (Salt & Hollick, 1944). Those collections provide more detailed information than has hitherto been available about the decline in the numbers of wireworms immediately following the breaking-up of grassland.

(2) *Qualitative.* All the larvae collected from the samples were carefully measured by means of a squared eye-piece in a binocular dissecting microscope. The measurements show the change in composition that accompanies the decline in numbers of the wireworm population.

(3) *Analytical.* From these quantitative and qualitative studies of the wireworm population, and from observations made for the purpose, some conclusions can be drawn about the factors responsible for the observed effects of cultivation.

SITES AND SAMPLING METHODS

The Secretary of the Cambridgeshire War Agricultural Executive Committee, Mr J. A. McMillan, kindly suggested five fields near Cambridge which, after being under grass for many years, were scheduled for ploughing in the first months of 1943. These fields are here called Boxworth, Swavesey 268, Swavesey 272, Lolworth, and Papworth. Their Ordnance Survey numbers, and information about their soil, cultivation and crop, will be found in the Appendix. Since they were all ploughed in the spring of 1943 and were sampled by us at similar periods during the succeeding months, they can be treated together as a group. They provide the bulk of the information we have to offer.

A sixth field, Swavesey 400, must be considered separately. It was broken up from rough grass and bushes by gyrotiller in August 1943 and is the only summer-ploughed field we were able to study while it was still under grass.

A field called Trinity I, on the University Farm, Cambridge, was ploughed from very old grass in August 1941, before we went on it. We sampled it in the spring and autumn of both 1942 and 1943, and so obtained a little useful information about the composition of its wireworm population in the second and third years of cultivation.

An eighth field, Spinney Pasture, provided information essential to this paper although it remained under grass throughout the period of our observations. This field, which has been rather fully described in another paper (Salt & Hollick, 1946),

was sampled at monthly intervals during 1942 and 1943, and therefore provides a standard with which the ploughed fields can be compared.

Samples were taken from all of these fields except the last in a manner similar to that of the National Wireworm Survey (Advisory Entomologists' Conference, 1944, p. 5), the twenty samples forming a set being distributed more or less evenly over the field. When two sets (forty samples) or three sets (sixty samples) were taken from a field on the same day, the field was traversed lengthwise four or six times, and ten samples were taken on each crossing. For purposes of tabulation and comparison these double or triple samplings have been separated into single sets of twenty samples, alternate samples in the one case, every third sample in the other, being assigned to separate sets.

The soil samples were collected by means of the ordinary boring tool, which removes a cylinder of soil 4 in. in diameter and 6 in. deep. We invariably carried our samples to a depth of 12 in. by reinserting the tool and removing a second core from underneath the first. The two cores, upper and lower, were put into different tins, and were examined separately, so that the number of wireworms in the two layers could be compared. The wireworms were extracted from these samples by means of our research method (Salt & Hollick, 1944), and the wireworms from each core were isolated and were preserved in a separate vial.

The decline in numbers

The five fields that provide most of our information were first investigated in late January, February, and very early March 1943, just before they were broken up. From three of them, Boxworth, Swavesey 268 and Swavesey 272, forty samples were taken; from the other two, Lolworth and Papworth, which are small fields of less than 10 acres, twenty samples. After it was ploughed, each field was sampled at intervals of about 8 weeks, so that by the end of 1943 four more collections had been made from each field. In one field, Boxworth, a sixth collection was made in 1943, but it can be considered a duplicate of the fifth. Four of the fields were sampled again early in 1944. As the population declined, double or triple sets of samples were taken, because the sampling error per core is proportionately greater with lower wireworm populations but is reduced by taking more cores (Finney, 1941, p. 286).

The result of our examination of these samples is set out in Table 1, where the date of collection from each field is shown, and where each entry in the body of the table indicates the number of wireworms found in a set of twenty samples.

It is evident from Table 1 that the number of wireworms extracted from the soil samples declined rapidly within the period of about a year covered by our observations. The greater part of the reduction took place in the weeks immediately after the fields were ploughed, so that the number collected at the second sampling was little more than half of that found when the fields were under grass. The decline continued, however, and the wireworms extracted from the fifth sampling, before

the end of 1943, were only one-quarter as numerous as those from the first sampling, 8 or 9 months earlier.

Even in pastures, the wireworm population of the soil decreases during the summer months, and these collections from ploughed fields must first be compared with collections taken in the same months from grassland. The comparison is made possible by our monthly collections from Spinney Pasture, and is shown graphically in Fig. 1.

As the figure shows, there was no significant decrease in wireworm numbers at Spinney Pasture between February and May 1943. But during the same period, the number of wireworms collected from the upper 12 in. of soil in the five ploughed fields declined to 56·6% of its original level. That reduction must be attributed in

TABLE 1. *Number of larvae, pupae and adults of* Agriotes *found in twenty soil samples, 4 in. diameter and 12 in. deep, collected on the date shown*

	1943										1944	
	1st collection (under grass)		Date ploughed	2nd collection		3rd collection		4th collection		5th collection		collection
Swavesey 268	19 Feb.	225	15 Mar.	3 May	155	7 July	106	13 Sept.	64	4 Nov.	52	20 Mar. 61
	5 Mar.	237		10 May	155	7 July	84	13 Sept.	59	4 Nov.	57	20 Mar. 51
												20 Mar. 68
Swavesey 272	5 Mar.	263	30 Mar.	3 May	150	28 June	130	2 Sept.	86	26 Oct.	54	23 Feb. 42
	5 Mar.	248		3 May	132	19 July	127	2 Sept.	67	26 Oct.	53	23 Feb. 49
												23 Feb. 53
Boxworth	22 Jan.	312	28 Feb.	19 Apr.	109	15 June	85	20 Aug.	92	14 Oct.	73	4 May 81
	19 Feb.	236		19 Apr.	144	15 June	105	20 Aug.	66	14 Oct.	66	4 May 68
										26 Nov.	60	4 May 98
										26 Nov.	78	
Lolworth	22 Jan.	179	16 Feb.	10 May	148	15 June	118	20 Aug.	57	14 Oct.	67	14 Apr. 71
												14 Apr. 46
Papworth	6 Mar.	242	2 Apr.	27 May	104	27 July	113	28 Sept.	38	15 Nov.	64	—
Mean date	19 Feb.		14 Mar.	4 May		2 July		3 Sept.		1 Nov.		30 Mar.
Av. no. in 20 samples	243		—	137		109		66		62		63
% of original no.	100		—	56·5		44·7		27·2		25·7		25·8

one way or another to the ploughing and the cultivation preliminary to a first crop. Between June and September, the collections from the ploughed fields continued to decline, but some part of this process may have been a seasonal effect and nothing to do with cultivation, for there was a sharp fall in the wireworm population at Spinney Pasture in the same period. But at Spinney Pasture, newly emerged larvae replaced the loss and the autumn collections, from September onwards, show a recovery of the population to its former level. That recovery is not shown in the cultivated fields which, instead, continue to give collections on the average only about one-quarter of their original figure.

It should be observed that we have not claimed that the wireworm population of the five cultivated fields was reduced within a year to 25% of its original level. We have merely stated that the number of wireworms collected from our samples, taken

to a depth of 12 in., decreased in that proportion. One reason for this caution is shown by our sampling of the sixth field, Swavesey 400.

A double set of samples was removed from Swavesey 400 on 2 August 1943, immediately before it was ploughed. One set yielded 191, the other 233 wireworms. It is likely that the average population of the field was considerably higher than the number represented by these collections, because this sampling was made at the time when the wireworm population under grass, as shown at Spinney Pasture, was at its lowest. The field was broken up by gyrotiller on 2 August. Two sets of samples collected on 23 September gave eighty-one and seventy-three wireworms; and two sets collected on 18 November gave thirty-eight and forty-nine wireworms. To the

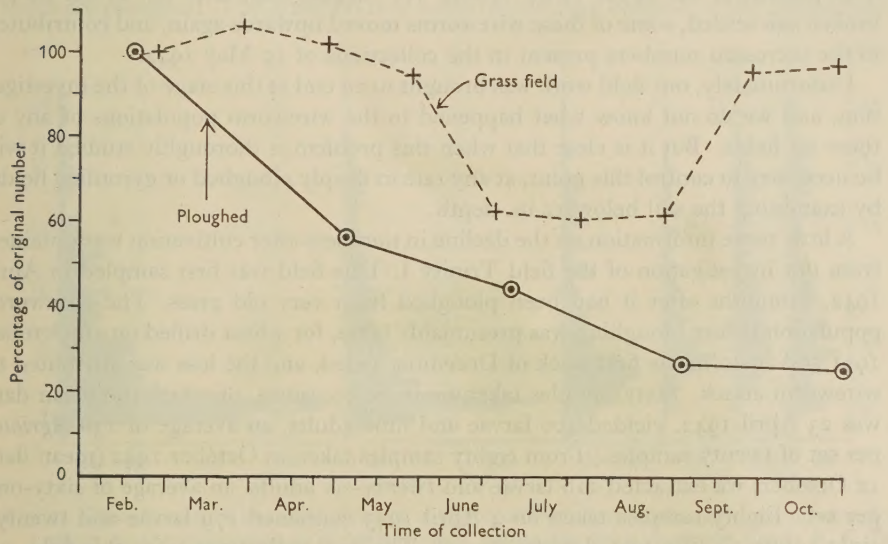


Fig. 1. The decline in the number of wireworms collected from five fields in the months immediately after ploughing, compared with the fluctuation in the number of wireworms collected from a grass field in the same months.

end of 1943, then, the decline at this summer-ploughed field exceeded the average decline at the five spring-ploughed fields, since the November samples included only 20% as many wireworms as the August samples, and perhaps less than 15% as many as they would have contained during the previous winter. Three sets of samples taken on 15 May 1944, however, contained 103, 103 and 114 wireworms; a recovery to 50% of the number collected in August.

This phenomenon is most marked in the collections from Swavesey 400, just described, but it is also apparent in those from some of the other five fields. The 1944 collections from Boxworth and Swavesey 268 both show it, and the fifth collections from Lolworth and Papworth both contained more wireworms than the fourth. Taking the five fields together, it appears that the phenomenon is to be

regarded less as a recovery than as an arrest of the decline, because on the average the 1944 collections are the same proportion of the original collections as are the fifth. But even this calls for explanation, and the very marked result at Swavesey demands it.

There can be no question of reinfestation of the fields to this extent during the winter, and the only suggestion that we are able to offer is that, following the cultivation of the field, a considerable proportion of the wireworm population of Swavesey 400 had moved so deep in the soil as to escape collection in samples carried to 12 in. depth. The collections on 23 September and 18 November, in that case, did not truly represent the total wireworm population, but only the population then present in the upper 12 in. of soil. We suggest that, later on, as the deeply broken soil settled, some of these wireworms moved upwards again, and contributed to the increased numbers present in the collections of 15 May 1944.

Unfortunately, our field work was brought to an end at this stage of the investigation, and we do not know what happened to the wireworm populations of any of these six fields. But it is clear that when this problem is thoroughly studied it will be necessary to control this point, at any rate in deeply ploughed or gyrotilled fields, by examining the soil below 12 in. depth.

A little more information on the decline in numbers after cultivation was obtained from our investigation of the field Trinity I. This field was first sampled in April 1942, 8 months after it had been ploughed from very old grass. The wireworm population before ploughing was presumably large, for wheat drilled on 16 October 1941 and again in the first week of December failed, and the loss was attributed to wireworm attack. Sixty samples taken on three occasions, of which the mean date was 23 April 1942, yielded 400 larvae and nine adults, an average of 136 *Agriotes* per set of twenty samples. From eighty samples taken in October 1942 (mean date 12 October) we extracted 216 larvae and twenty-six adults, an average of sixty-one per set. Eighty samples taken on 4 April 1943 contained 179 larvae and twenty-eight adults, or fifty-two *Agriotes* per set. The final collections from this field, on 4 and 7 October 1943, again totalled eighty samples and contained only forty-nine larvae and eight adults, an average of fourteen per set of twenty samples.

These collections from Trinity I are interesting in two respects. They show first that between April and October 1942, the year after ploughing, the number of wireworms collected from twenty samples taken to a depth of 12 in. decreased from 136 to sixty-one; and that between April and October 1943, the second year after ploughing, the number further decreased from fifty-two to fourteen. They also show, however, that between October 1942 and April 1943, there was comparatively little reduction, from sixty-one to fifty-two *Agriotes* per set of samples. This second point, together with the evidence referred to above (p. 173) from five other fields, suggests that the greater part of the decline in the numbers of wireworms after cultivation occurs during the summer months, and that during the winter the population of wireworms in the upper 12 in. of soil remains relatively constant.

THE CHANGE IN COMPOSITION

In a previous paper (Salt & Hollick, 1944), it was shown that a normal wireworm population under old grass is composed of very large numbers of small larvae and gradually decreasing numbers of the medium-sized and larger larvae; in short, that there is an inverse correlation between the number and size of the larvae forming the population. With that description, the wireworm populations of the five fields (Swavesey 268 and 272, Boxworth, Lolworth, and Papworth) conformed, before the fields were ploughed. Indeed, histograms illustrating the fact were published in support of the original demonstration (Salt & Hollick, 1944, fig. 7).

The question arises whether wireworm populations retain this composition after fields are ploughed. The question is of some importance because, if the composition

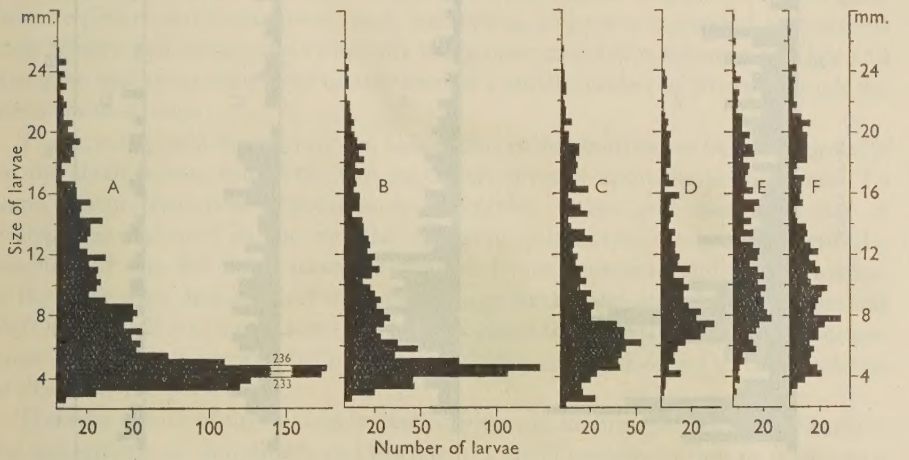


Fig. 2. Composition of the collections of wireworms from five fields: A, from 160 samples taken on the mean date 19 February 1943, before the fields were ploughed; B, C, D, from 160 samples taken on each of the mean dates 4 May, 2 July, 3 September, respectively, after the fields were ploughed; E, from 200 samples taken on the mean date 1 November; F, from 220 samples taken on the mean date 30 March 1944.

is changed, the alteration must be due to the effects of cultivation bearing more heavily on one part of the population than on another; and knowledge of that matter might be turned to account in control.

The answer is most clearly shown by means of the series of histograms in Fig. 2. In order to represent the situation before ploughing, the lengths of the 1901 larvae collected (together with forty-two adults) from the five fields at their first sampling are plotted in Fig. 2A. That histogram is clearly of the normal form for grassland populations, with a large proportion of very small larvae. The second lot of samples contained 1083 larvae and fourteen adults, and the lengths of the larvae take the form of Fig. 2B. The histogram again has a triangular shape, but is much narrowed,

especially by the great reduction in the number of larvae 3–6 mm. long. The third collection included 852 larvae, fourteen pupae and two adults. The histogram representing the larvae, Fig. 2C, presents a greatly altered appearance. Wireworms 5–7 mm. long are the most numerous, but there is no longer an overwhelming preponderance of small ones. By the mean date of the fourth collection, 3 September, the composition of the collected population was noticeably different. The samples contained 489 larvae and forty adults, and the lengths of the larvae are plotted in Fig. 2D. The most numerous groups of larvae are those between 7 and 8 mm., and larvae shorter than 6 mm. long are few, so that the diagram is not broadly based,

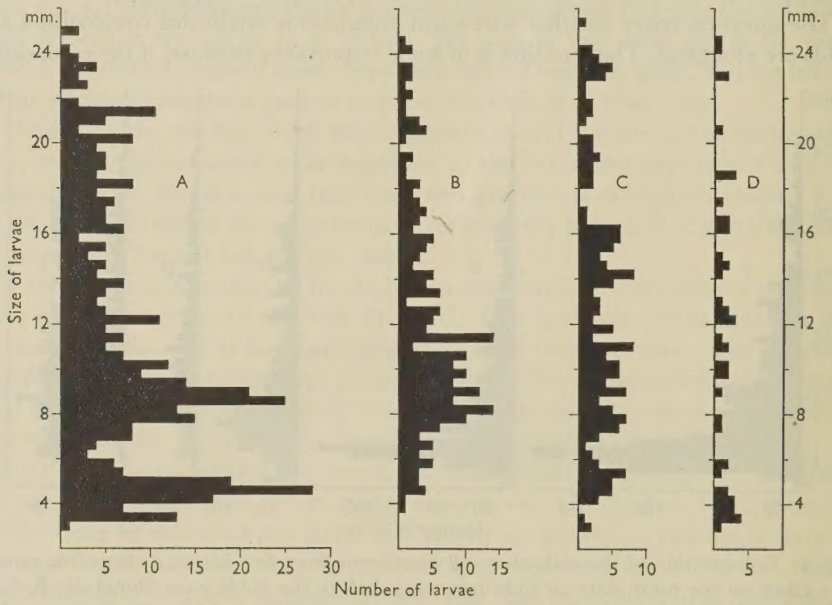


Fig. 3. Composition of the collections of wireworms from the field Trinity I, ploughed from old grass in August 1941: A, in April 1942; B, in October 1942; C, in April 1943; D, in October 1943 (A, sixty samples; B, C, D, eighty samples each).

like those of the first and second collections, but tapers downwards at its base. The last sampling of the year consisted of ten instead of eight sets of samples, and contained 561 larvae together with sixty-three adults. The lengths of the larvae, as plotted in Fig. 2E, can no longer be said to take a triangular shape. The same is true of the final histogram, Fig. 2F, representing the lengths of 627 larvae which, with sixty-one adults, were obtained from eleven sets of samples collected about the mean date 14 March 1944.

No further collections were made from these fields, but later stages of the change in population structure can be observed in our records of Trinity I. The four collections from this field have been described above (p. 174), and the larvae

included in them are represented in Fig. 3. The four histograms illustrate the wireworm population of Trinity I in April and October of the second and third years after ploughing. The first, Fig. 3 A, shows that the 400 larvae collected in April 1942 form a relic of what we assume to have been originally a normal population pyramid. The second and third, Fig. 3 B, C, represent the collections of October 1942, and April 1943, and illustrate the disappearance of the pyramidal form. The fourth, Fig. 3 D, represents the collection of October 1943, 2 years and 2 months after the field was ploughed, and shows the depleted population composed of a small number of larvae distributed rather uniformly among the different size-groups.

The two series of histograms show that the decline in numbers of wireworms after cultivation was accompanied by a striking change in the composition of the population. The broadly based triangle of the wireworm population under grass became progressively narrower; each succeeding collection contained proportionately fewer small larvae; and eventually the inverse correlation between number and size gave way to a rather even distribution of a small number of larvae through the various size-groups.

The first impression given by the histograms is that cultivation bore very heavily on the small larvae, and acted with much less severity upon the larger larvae. To some extent, that first impression is deceptive. Other processes than that of destruction operated in the months following cultivation. Oviposition and the hatching of eggs led to an accession of small larvae; pupation and transformation to the adult stage drained away some of the large larvae; and growth caused a general shift from smaller to larger size-groups throughout the population. These processes must be taken into account before a balanced view can be obtained of the incidence of death on the population.

The true nature of the change in composition can be brought out more clearly if the size-groups are simplified, and the fate of a small number of groups is followed in detail. With that intention, we have divided the collections into three: small wireworms between 2·1 and 8 mm. long, medium-sized wireworms between 8·1 and 14 mm. long, and large ones over 14 mm. long. The numbers forming each of those groups in the six collections are given in Table 2, but the processes we wish to trace are more easily followed in Fig. 4 where, for each group of larvae, there has been plotted the percentage in each collection of the number found in that group at the first sampling.

Between the first and second collections all three groups of larvae were much reduced in number. The small larvae appear to have been most affected. Since, however, a good deal of larval growth probably took place between February and early May, the groups of medium-sized and large larvae may each have received an increment from the group next smaller. Any correction for this process of growth would show a greater reduction in the number of large larvae; probably, since the numbers are larger, an increase in the reduction of the medium-sized larvae; and a decrease in the apparent loss of the small larvae.

In the second period, between the mean dates of 4 May and 2 July, the hatching of eggs added a few larvae of the smallest size-groups to the collection, as is clearly seen by comparing Fig. 2B and 2C. Their numbers must have been small, however, for there were only seventy-six larvae less than 4 mm. long in the third collection, and some of those may have been laggards of the original small larvae. At the other

TABLE 2. *Number of wireworms of different sizes found in eight sets each of twenty soil samples*

		Small larvae		Medium-sized larvae		Large larvae		All larvae	
	Collection	Number	Percentage	Number	Percentage	Number	Percentage	Number	Percentage
1	19 Feb.	1237	100	416	100	248	100	1901	100
2	4 May	643	52	273	66	167	67	1083	57
3	2 July	454	37	219	53	179	72	852	45
4	3 Sept.	183	15	196	47	110	44	489	26
5	1 Nov.	122*	8	235*	45	204*	66	561*	24
6	30 Mar.	206†	12	240†	42	181†	53	627†	24

* In ten sets of samples. † In eleven sets of samples.

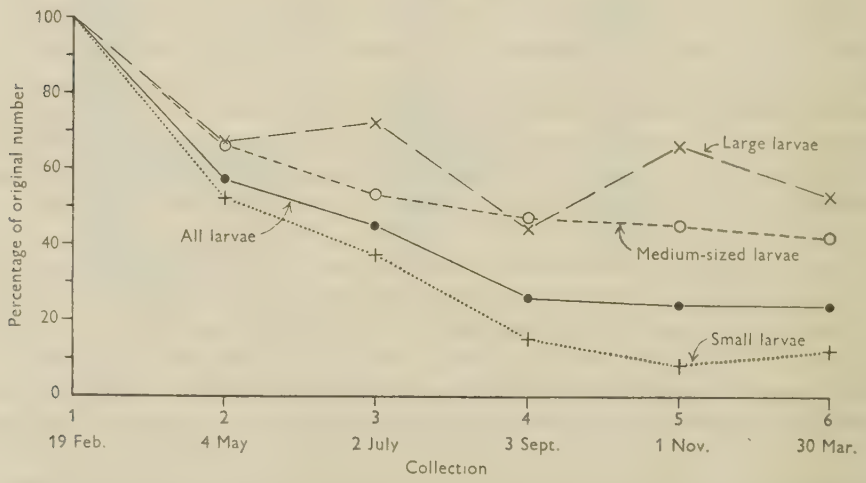


Fig. 4. Small (2·1–8 mm.), medium-sized (8·1–14 mm.), and large (over 14 mm.) wireworms collected from five fields on six occasions, shown as the proportion of the original number in each group. The fields were ploughed from grass between the first and second collections.

end of the scale, the third collection included fourteen pupae which must, and two adults which might, have developed from large larvae after the date of the preceding samples. In spite of this loss, the large larvae actually increased in number in the period under consideration. That increase can only have been due to the growth of larvae from the medium-sized group during the months of May and June. Some of

the decline in numbers of the middle group, therefore, must certainly have been due to growth. On similar grounds, although the evidence is hidden, it is likely that some of the reduction of the small larvae was due to growth into the medium-sized group.

During the months of July and August, between the third and fourth collections, all three groups of larvae decreased in number. The rapid decline of the large larvae reflects pupation and transformation, for the forty adult beetles in the fourth collection must all have developed from large larvae during the summer of 1943. But this same group lost sixty-nine individuals during the period so that, even without allowing for any increment due to growth of medium-sized larvae, one in six of the large larvae at the beginning of the period was destroyed (or disappeared from the population sampled) within these 9 weeks.

The period between the fourth and fifth collections was marked by an increase in the number of large larvae, which can be attributed to growth during the late summer feeding period. The increment came from the medium-sized group which, in turn, since it shows a reduction less than that increment, must have been maintained by the group of small larvae. Which group contributed most to the total loss of forty larvae, it is impossible to say, but certainly the graph must not be read as indicating that only small larvae were destroyed in this period.

During the winter months, the group of large larvae declined which, since we have no evidence of negative growth, argues for the destruction of large larvae during the period. The small larvae were actually more numerous in the sixth collection than in the fifth. This was due principally to an increased number of larvae between 4 and 5 mm. long. Where they came from, if not from the soil below 12 in. deep, we cannot suggest. Since *Agriotes* adults lay their eggs in clutches, it might be supposed that the sixth collection included by chance a number of samples containing large clutches of undispersed small larvae, but our detailed records show that no such explanation is tenable.

This account, together with Fig. 4, makes it clear that the number of large larvae in the upper 12 in. of soil declined at three periods during the first year of cultivation. It shows how that decline was obscured by increments of medium-sized larvae growing up to be classified as large larvae. It also emphasizes, what is perhaps evident when clearly stated, that the process of growth necessarily exaggerates the decrease in numbers of the small larvae and hides the decline of the large larvae.

The change in composition of the wireworm population after cultivation can be summarized as a change from a population predominantly of small larvae to one composed of more uniform numbers of the different size-groups. That change, however, is not to be thought of as being brought about simply by the destruction of the small larvae and the comparative immunity of the large larvae. It is true that large numbers of small larvae disappear in the year after ploughing, but it is also true that far more of the medium-sized and large larvae are lost than appears from a simple comparison of the populations at the beginning and end of the period.

Factors responsible for the effects of cultivation

Three principal groups of factors might be responsible for the progressive decline in the number of wireworms in the years following the ploughing-up of grassland: (1) An arable field is subjected to successive agricultural operations which might cause the immediate destruction of wireworms through mechanical injury or by exposing them to birds and other predators. (2) The physical condition of cultivated land might be unsuitable for high wireworm populations; either directly, through its different texture or composition, or indirectly, through changes in the flora and other fauna. (3) Wireworm adults might fail to oviposit, or oviposit at a much lower rate, on cultivated ground, so that the population would decline for want of replacement.

The separate effects of these three groups of factors could be distinguished by regular sampling of fields over a period of years. If the low wireworm populations of arable land were due entirely to the actual processes of cultivation, the decline would take the form of a number of sudden falls synchronized with those processes, the population remaining relatively constant between successive periods of cultivation. If the reduction were due only to the failure of the population to replenish itself, the course of the decline should again be a series of steps, in this case annual and synchronized with the period of pupation. If the reduction were due only to the continuous effect of a changed physical condition of the soil, it might be expected that the decline would be gradual, not in sharply marked steps, although it might be slowed or arrested during the winter months. Each of these three possibilities can now be examined with reference to our data.

(1) *Immediate effects of cultivation*

There can be no doubt that the process of cultivation has an immediate and considerable effect on the wireworm population. Our most direct evidence comes from observation on the field Trinity I. Following the failure of winter wheat, this field was cultivated on 23 April 1942 by means of disk and drag harrows drawn by a tractor. While the machines were still at work, and again later on the same day, we made collections of wireworms and examined their condition. Since the collections were made in the open, and by eye, the wireworms found were all rather large; none being shorter than 8 mm., and most of them being longer than 10 mm.

Taking first an area near the middle of the field, we searched the soil immediately after the machines had passed over it and collected eighty wireworms, of which sixty-one appeared to be healthy, fifteen were so broken or crushed as to be either dead or moribund, and four were in doubtful condition. These last were isolated in four vials of soil. The next day two were dead, the other two were alive and were recorded as healthy. Of the eighty larvae found, then, seventeen were fatally injured and sixty-three were unharmed.

A similar collection was made on an area at the edge of the field immediately after

the tractor and the implements had turned on it. In that place, 165 wireworms were found, of which, after the doubtful ones had been kept isolated for 24 hr., 138 were recorded as healthy and twenty-seven were broken or crushed.

In the evening, between 1 and 4 hr. after the harrowing was finished, the soil was carefully examined to the depth of cultivation in an area 3 ft. square near the middle of the field. There, 134 larvae were found, of which 119 appeared healthy and fifteen were injured. At the edge of the field, an area 4 ft. by 3 ft. was similarly examined to the depth of cultivation. In that place 155 larvae were found, of which 111 seemed to be healthy and forty-four proved to be fatally injured.

In summary, of a total of 214 larvae found inside the field, thirty-two were injured so severely that they could not survive; at the edge of the field, within the headland on which the implements were turned, 320 larvae were collected, of which seventy-one were fatally injured. In these particular collections, then, 15% of the wireworms in the interior of the field and 22% of those in the headlands were destroyed by a normal process of cultivation.

To the number of wireworms destroyed directly by farm implements must be added those that are exposed by the cultivation to destruction by predators, especially birds. That birds following the plough eat wireworms that are turned up has been known a long while, and an extensive literature (cf. Thomas, 1940) has grown up on the extent of this destruction. Where the estimates are based on the contents of birds' crops, it should be borne in mind that a proportion of the wireworms in the crops had probably already been injured by the process of cultivation.

The decline of the wireworm population consequent upon these immediate effects of cultivation is shown in our graphs (Figs. 1 and 4), where the great reduction between the first and second collections must be attributed largely to the process of breaking-up from grassland. To trace the effect of later cultivation, however, would be much more difficult, for the several fields were tilled by such different methods and at such widely separated dates that each field, and in some cases different parts of the same field, would have to be considered separately. Fortunately, in view of the direct evidence we have recorded above, that indirect evidence of the effect of cultivation is unnecessary.

(2) *Physical conditions of cultivated soil*

How far the physical condition of cultivated soil adversely affects the wireworm population is difficult to determine. The distinguishing mark of this effect would be a gradual and continuous decline of the population, perhaps accelerated after periods of cultivation. Table 1 and Fig. 1 show that there was a decline of the *Agriotes* population in our fields between the second and third collections, and between the third and fourth, although only at the small Lolworth field and on part of Swavesey 268 did any cultivation take place during this time. That decrease of 229 and 339 individuals in the two periods, when corrected for the appearance of newly hatched larvae in the earlier period and for the pupation of large larvae in the

later, can be attributed to natural mortality in arable land. The problem remains whether the decline is greater in these cultivated fields than it is under grass. Unfortunately, our work on seasonal changes in the population at Spinney Pasture, which can eventually be used as a yard-stick for this purpose, is still not completed and the comparison we should like to make is not yet possible.

(3) *Failure of replenishment*

The hatching of *Agriotes* eggs takes place in Cambridgeshire principally in June and July. The effect of this on a normal wireworm population is illustrated in Fig. 5A. The diagrams represent the collections of small larvae found at Spinney

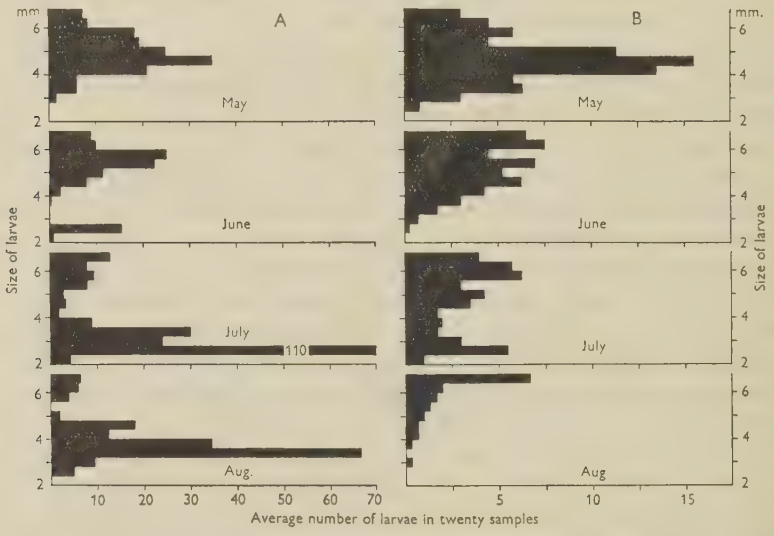


Fig. 5. Size distribution of the smaller wireworms collected in May, June, July and August: A, from a grass field; B, from five fields ploughed from grass on the mean date 14 March.

Pasture in the months of May, June, July and August, of the two years 1942 and 1943; each histogram including the collections from forty samples. In the May collections there were no larvae of the smallest size-groups. Eggs were found in the June samples as well as a number of small larvae. In the July samples there were large numbers of very small wireworms, which must have emerged from recently laid eggs; and this group of larvae is clearly illustrated, although the larvae have grown larger, in the diagram of the August collections.

The development of the wireworm population in the five ploughed fields took a different course. In May, 120 samples were taken from one or more of these fields (Table 1), in June eighty, in July eighty, in August sixty. When we examine the population-size diagrams of these collections (Fig. 5B), we find that in May they

resemble the collections from Spinney Pasture in having no members of the smallest size-groups. In June, July and August, however, they are very different. The large numbers of very small larvae that appeared in the June collections at Spinney Pasture are here lacking. A few small larvae, presumably newly hatched, are present, but their numbers are trivial in comparison with those from the grassland samples.

The absence of these small larvae is also shown by the collections from the older arable field, Trinity I, which was sampled in April and October 1942 and in the same months in 1943. There is little sign in the October collections of either year (cf. Fig. 3) of the great influx of small larvae that is so marked a feature of autumn collections under grass.

This failure of the population to replenish itself may have been due in part to the destruction, as large larvae or pupae, of many of the individuals on which egg-laying depended. It may also have been due to the failure of adult beetles to oviposit freely in cultivated fields, or to an excessively high death-rate of eggs and very young larvae under the conditions of cultivated soil. How it was brought about, however, need not concern us at present. The important fact is clear: that young larvae, on which the population depends for replenishment, occurred in the arable fields investigated only in very reduced numbers.

At the beginning of this section we suggested that three main groups of factors might be responsible for the progressive decline of the wireworm population following the ploughing-up of grassland. Two of them, the immediate effects of cultivation and the failure of the wireworm population to replenish itself in arable land, have been shown to have operated in the fields we studied. The effect of the third, the physical condition of cultivated soil, has not been satisfactorily demonstrated, because with our present information we cannot clearly distinguish its extent; but there was in the fields investigated a reduction in wireworm numbers which may have been due to that cause.

Of these three groups of factors, the one most worthy of active study, from an economic point of view, is that including the immediate effects of cultivation. These effects can be hastened and intensified; the others we have discussed cannot. Direct destruction of wireworms by the normal processes of cultivation seems from our observations to offer a possibility of control. It remains for those with the necessary facilities to show by what agricultural operations and at what season the maximum destruction of wireworms can be produced with the least effort.

Discussion

Most of the observations recorded above were made in 1942 and 1943, and some of them were made known to advisory entomologists and others in a mimeographed report circulated in September 1942. It is therefore not surprising that a method of approach we outlined so long ago has been reflected in other investigations, and that some details of our work have been repeated and already published. Perhaps it calls

for apology that the demands of our proper duties have delayed this account so long.

The present paper is merely a contribution to a large problem that is still scarcely explored, much less solved; and an elaborate discussion of the literature would be out of proportion. We should like, however, to mention the impressive series of four papers published by Stapley and his co-workers, two of which concern us here. The latest of them (Stapley, Ross & Cockbill, 1947) describes the changes in wireworm populations during a bare fallow, and, so far as that paper and this deal with comparable subjects, they are in excellent agreement.

That cannot be said for the third paper of the series (Cockbill, Ross & Stapley, 1947), which deals with population changes after summer ploughing, with reference to six fields in Huntingdonshire. Since the fields sampled in that investigation were only a few miles from those we studied and the period, late 1943 and 1944, partly coincident with that in which we worked; and since samples were taken to a depth of 12 in. and were examined by a method estimated to yield about 95% of the larvae present, the results ought to be fairly comparable with ours. Yet these workers reach the conclusion (p. 87), 'It seems unlikely, therefore, that ploughing has any immediate effect on the population.'

This statement seems so completely at variance with observations we have recorded above that some explanation is called for. Of several suggestions that might be offered, we confine ourselves to pointing out that Stapley and his collaborators were here dealing exclusively with fields ploughed in the summer of 1943; while our work had to do mainly with fields broken up in the preceding spring. It might be, therefore, that early ploughing affected the wireworm population so much more than summer ploughing as almost entirely to explain the apparent contradiction. That suggestion would find support in the statement of Stapley *et al.* (1947, p. 101), 'the results indicate that the reduction in population is greater on the earlier ploughed plots and especially that there is a marked difference between the plots ploughed before and after July'.

To such terms of truce our observations on Swavesey 400, the only field we studied that was broken up in summer, remain opposed. But it is to be noticed that the results obtained by the other workers on two of their six fields, namely on fields VIII and X, are not very dissimilar from ours at Swavesey 400. Perhaps in some soils, or following some forms of cultivation, there is an immediate effect of summer ploughing.

Indeed, in spite of the striking statement that has led us to this discussion, the difference between the two investigations may be one of detail rather than principle. A more qualified conclusion published later in the same paper (Cockbill *et al.* 1947, pp. 92–3) is unfortunately too long for full quotation, but begins: 'If the results we have obtained are examined against the background of the grass "controls" a different picture is obtained.' And goes on: 'Therefore, a net decline of considerable magnitude has taken place.' This is much more compatible with our observations.

Whether the results of the two investigations are fundamentally opposed, or basically in agreement although perhaps divergent in detail, their differences emphasize the need for further work on this problem. In such preliminary studies as these, seeming contradictions do not necessarily mean that one group of workers s rig ht and another group wrong. Each has dealt with certain factors and, out of the welter of natural conditions, has had to neglect others. Different results at this stage, therefore, are not only to be expected but even to be welcomed, for in seeking an explanation of their diversity the essential factors may be brought to light.

We are indebted to Prof. J. Gray, F.R.S. for laboratory facilities; to the Agricultural Research Council for a grant which provided materials, transport and assistance, and to Mr J. A. McMillan and his staff on the Cambridgeshire War Agricultural Executive Committee for allowing us access to fields under their charge.

APPENDIX

Abbreviated notes on the fields

Boxworth, O.S. 152

5·6 acres. Loam on sandy clay. Grass for at least 25 years. December 1942, mole drained at 22 in. 28 February 1943, ploughed, disked. 15 March, rolled, disked three times. 22 April, sown flax, rolled. 2 August, flax pulled. 8 November, ploughed.

Swavesey, O.S. 268

23·5 acres. Loamy clay over heavy clay. Grass for many years. 9 March 1943, mole drained. 15 March, ploughed. 21 April, disked twice, rolled twice. 4 May, 5 acres drilled kale, rolled. 8 May, 18½ acres drilled flax, harrowed. 11 May, rolled. 16 June, kale failed, 5 acres disked twice, rolled. 18 June, 5 acres drilled kale. 10 July, second kale failed, 5 acres ploughed. 31 August, 18½ acres flax pulled. 3 October, 18½ acres ploughed.

Swavesey, O.S. 272

24·9 acres. Heavy clay. Grass for at least 20 years. 10 March 1943, mole drained. 30 March, ploughed. 22 April, disked twice, rolled twice. 7 May, drilled peas, harrowed. 8 May, rolled. 23 August, cut. 24 September, ploughed. 2 October, disked, drilled wheat, harrowed. 4 October, harrowed.

Lolworth (Swavesey, O.S. 444)

7·1 acres. Clay with gravel. Rough grass with poor drainage. 28 January 1943, mole drained and main leads piped. 16 February, ploughed. 12 March, disked twice. 18 March, rolled. 15 April, drilled flax, harrowed, rolled. (Early) August, flax pulled. 24 August, ploughed, cultivated once. 16 September, heavy harrow once over. 6 October, drilled oats, harrowed.

Papworth St Agnes, O.S. 47

6·8 acres. Light loam with some clay and chalk below. Grass for about 20 years. 2 April 1943, ploughed. 5 April, rolled. 12 April, disked twice, rolled. 14 April, disked twice, rolled. 15 April, sown flax. 17 April, rolled. 28 August, flax pulled. 10 September, cultivated twice. 8 October, ploughed.

Swavesey, O.S. 400

8·6 acres. Heavy clay. Rough pasture with many small hawthorn bushes. 2 August 1943, broken up by gyrotiller. No record of further cultivation and on 18 November 1943, at the third sampling, the field appeared to have been untouched since it was broken up. During April 1944 flax was drilled.

REFERENCES

Advisory Entomologists' Conference (1944). Wireworms and Food Production. *Bull. Minist. Agric., Lond.*, no. 128, 64 pp.

Cockbill, G. F., Ross, D. M. & Stapley, J. H. (1947). Wireworm populations in relation to crop production. III. Population changes after summer ploughing. *Ann. appl. Biol.* **34**, 83–94.

Finney, D. J. (1941). Wireworm populations and their effect on crops. *Ann. appl. Biol.* **28**, 282–95.

Roebuck, A. (1924). Destruction of wireworms. *J. Minist. Agric.* **30**, 1047–51.

Salt, G. & Hollick, F. S. J. (1944). Studies of wireworm populations. I. A census of wireworms in pasture. *Ann. appl. Biol.* **31**, 52–64.

Salt, G. & Hollick, F. S. J. (1946). Studies of wireworm populations. II. Spatial distribution. *J. exp. Biol.* **23**, 1–46.

Stapley, J. H., Ross, D. M. & Cockbill, G. F. (1947). Wireworm populations in relation to crop production. IV. Population changes during a bare fallow. *Ann. appl. Biol.* **34**, 95–103.

Thomas, C. A. (1940). The biology and control of wireworms. *Bull. Pa. agric. Exp. Sta.* no. 392, 90 pp.

(*Received* 8 *July* 1948)

THE ADJUSTMENT FOR A NATURAL RESPONSE RATE IN PROBIT ANALYSIS

By D. J. FINNEY

Lecturer in the Design and Analysis of Scientific Experiment, University of Oxford

A revised scheme of computation is suggested for the fitting of a probit regression line to quantal response data which have to be adjusted because of the occurrence of natural responses not caused by the stimulus under test. The calculations lead to the same results as those proposed when the method was first introduced, but have the advantages of close similarity with multiple regression calculations and of simplifying the test of heterogeneity. The new scheme is illustrated on the example used in the earlier paper.

A table of weighting coefficients for use with high natural response rates is presented.

1. Introduction

A few years ago (Finney, 1944), the writer introduced a method of allowing for a natural response rate, independent of the effect of any stimulus under examination, in the standard calculations for estimating a probit regression line. The need for this arises, for example, in tests of the toxicity of an insecticide: between the time of application of the insecticide and the time of examination of the insects, some insects may die from 'natural causes', unconnected with the treatment whose effect is to be assessed, and evaluation of the insecticide requires that the irrelevant effects of natural mortality be first removed.

In these circumstances, not only must each observed response rate be adjusted to allow for the natural rate, but, as is shown by mathematical theory, the weight to be attached to observations must be reduced because not all the subjects tested are available for assessment of the potency of the stimulus. If the natural response rate is known, the requisite changes are quite simple, and involve no new features in a probit analysis. More commonly, however, the natural rate must itself be estimated from the data, and additional steps must then be incorporated into the calculations. A scheme of computation has been described, with numerical examples, in earlier publications (Finney, 1944; 1947, § 28), and tables of the modified weighting coefficient have been provided. Recent research now in preparation for publication (Finney, 1949) has shown that the solution of the problem is a particular case of a general theorem on the estimation of the distribution of individual tolerances, and has indicated an improved scheme of computation. This scheme has the advantage of following the standard pattern for multiple linear regression; it is described in the next section, and illustrated by use on the example discussed in the previous paper.

Horsfall (1945) has severely criticized the previous work on this problem, contending that its logical basis is inappropriate to the facts. He says that the correction of observed percentage responses by Abbot's formula, at least in toxicological tests, 'appears to involve the assumption that all the individuals dying "naturally" are normally distributed with regard to the log dose of the toxicant. It seems more logical to suppose that they represent weaklings and that they comprise that segment of the frequency distribution that is most readily killed by the toxicant....Finney (1944) has recently looked into the problem. He recognized that Abbott's formula is valid only "when the poison and the natural mortality operate independently: any interaction between the two causes of death destroys its validity". Assuming no interaction, Finney then set forth to erect a gorgeous structure of weighting coefficients to improve the estimate of error. It seems unfortunate that he did not first attempt to make an improvement on Abbott's correction before expending so much effort based on the assumption that it is accurate.'

What Horsfall means by a normal distribution of natural deaths with regard to the log dose of the toxicant is not clear; the assumption underlying the analysis proposed in 1944 is that the *tolerances* (or doses just sufficient to cause death) of individuals for the toxicant were normally distributed on a logarithmic scale and that, independently of the dose, a proportion of individuals would die from natural causes (that proportion varying from dose to dose solely in accordance with the binomial distribution). The assumption of a normal log tolerance distribution may be false, and needs experimental investigation; nevertheless, it follows the common practice of assuming the distribution of biological measurements to be approximately normal, unless there is definite theoretical or experimental evidence to the contrary. Its consequences are likely to be no more serious than an assumption of normal distribution for heights or weights, and it is surely more reasonable than Horsfall's suggestion of an excess of easily killed weaklings (which would imply a distribution of individual tolerances of some peculiar form). Indeed, the proposal to regard 'natural mortality' as 'weakling mortality' is manifestly false for the numerical example discussed in the 1944 paper (and in other data since examined). In that ovicidal trial, 56% of untreated eggs failed to hatch, whereas with doses of 0·0025 and 0·005% pyrethrins 63 and 65% respectively failed to hatch: clearly most, if not all, failures at low doses were of eggs that would not have hatched even had they been untreated, and not of 'weaklings' that were easily destroyed by a very small dose (cf. similar, though less striking, results for an insecticidal test using derris: Finney, 1947, § 28). It is possible, of course, that the probability of an insect or a fungus spore meeting with death from natural causes may be correlated with its tolerance of a toxicant under test, and this would complicate the mathematical model of the situation. Serious disturbance from this cause would be likely to lead to systematic deviation of data from equations fitted by the statistical technique previously recommended, and the non-occurrence of such deviations suggests that the technique is not far wrong for the data to which it has been applied. Almost every

method of statistical analysis that is applied to biological data involves an assumption that the data possess certain properties in common with a precise mathematical model, an assumption which the investigator usually knows to be not exactly true but believes, on the basis of past experience, to be an approximation sufficiently close to be able to give conclusions of practical value. Despite Horsfall's criticisms, the writer sees no reason to doubt that the technique he proposed is, in general, as near the truth as is any other method based on mathematical theory but used for observational data. In any case, the occurrence of an appreciable proportion of responses not directly attributable to the applied treatments must decrease the weight to be attached to the observations on percentage mortality, and at worst the 'gorgeous structure of weighting coefficients' will be nearer the truth than the ordinary probit weights. Improvement and modification may be desirable for some types of data, but the next step should be experimental demonstration of the phenomena rather than mathematical investigation of the consequences of deviation from the simplest theory.

2. Scheme of computation

In order to simplify the terminology, the methods will be described here as applied to insecticidal toxicity test data, though obviously the statistical technique is of much wider applicability. Suppose that C is a first estimate of the natural mortality rate for the data, based upon observation of 'control' or untreated subjects or from general inspection of the data. If a batch of n subjects receives a certain dose, and r of them die, the observed death rate, p^*, is

$$p^* = r/n, \tag{1}$$

and the proportion actually killed by the poison may be estimated from Abbott's formula as

$$p = \frac{p^* - C}{1 - C}. \tag{2}$$

If C were known exactly, the probit analysis of the data would be calculated from values of p for different doses, in the usual manner except for a modification of the weighting coefficient to

$$w = \frac{Z^2}{Q\{P + C/(1-C)\}} \tag{3}$$

(Finney, 1944). Values of w have been tabulated by Finney (1947) and Fisher & Yates (1948), for values of C up to 40%. Table 1 extends Fisher and Yates's simplified table up to 95%, and a more detailed table is available in manuscript.

If C is not known exactly, an adjustment (δC) to the first estimate must be calculated. The adjustment may most easily be found by introducing t, an auxiliary variate defined by

$$t = Q/Z, \tag{4}$$

numerical values of which are shown in Table 1 and also in earlier tables.

TABLE 1. *Weighting coefficients for use when natural mortality occurs*

		Percentage natural mortality										
Y	*Q*/*Z*	45	50	55	60	65	70	75	80	85	90	95
2·6	44·288	0·001	—	—	—	—	—	—	—	—	—	—
2·7	34·923	0·001	0·001	0·001	0·001	—	—	—	—	—	—	—
2·8	27·797	0·002	0·001	0·001	0·001	0·001	0·001	—	—	—	—	—
2·9	22·330	0·002	0·002	0·002	0·001	0·001	0·001	0·001	—	—	—	—
3·0	18·101	0·004	0·003	0·002	0·002	0·002	0·001	0·001	0·001	0·001	—	—
3·1	14·802	0·005	0·004	0·004	0·003	0·002	0·002	0·001	0·001	0·001	—	—
3·2	12·211	0·008	0·006	0·005	0·004	0·003	0·003	0·002	0·002	0·001	0·001	—
3·3	10·159	0·011	0·009	0·007	0·006	0·005	0·004	0·003	0·002	0·002	0·001	—
3·4	8·521	0·015	0·012	0·010	0·008	0·007	0·005	0·004	0·003	0·002	0·001	0·001
3·5	7·205	0·020	0·017	0·014	0·011	0·009	0·007	0·006	0·004	0·003	0·002	0·001
3·6	6·1394	0·027	0·023	0·019	0·015	0·013	0·010	0·008	0·006	0·004	0·003	0·001
3·7	5·2705	0·036	0·030	0·025	0·020	0·017	0·013	0·010	0·008	0·006	0·004	0·002
3·8	4·5571	0·046	0·038	0·032	0·026	0·022	0·017	0·014	0·010	0·007	0·005	0·002
3·9	3·9676	0·058	0·048	0·040	0·034	0·028	0·022	0·018	0·013	0·009	0·006	0·003
4·0	3·4770	0·071	0·060	0·050	0·042	0·035	0·028	0·022	0·017	0·012	0·008	0·004
4·1	3·0665	0·087	0·073	0·062	0·052	0·043	0·034	0·027	0·021	0·015	0·009	0·005
4·2	2·7206	0·103	0·088	0·074	0·062	0·051	0·042	0·033	0·025	0·018	0·012	0·006
4·3	2·4276	0·121	0·104	0·088	0·074	0·061	0·050	0·040	0·030	0·022	0·014	0·007
4·4	2·1780	0·140	0·120	0·102	0·086	0·072	0·059	0·047	0·036	0·026	0·016	0·008
4·5	1·9640	0·159	0·137	0·117	0·099	0·083	0·068	0·054	0·042	0·030	0·019	0·009
4·6	1·7797	0·178	0·154	0·132	0·112	0·094	0·077	0·062	0·048	0·034	0·022	0·011
4·7	1·6202	0·196	0·170	0·147	0·125	0·105	0·087	0·070	0·054	0·039	0·025	0·012
4·8	1·4814	0·213	0·186	0·161	0·137	0·116	0·096	0·077	0·060	0·043	0·028	0·014
4·9	1·3599	0·228	0·200	0·174	0·149	0·126	0·104	0·084	0·065	0·048	0·031	0·015
5·0	1·2533	0·241	0·212	0·185	0·159	0·135	0·112	0·091	0·071	0·052	0·034	0·016
5·1	1·1593	0·252	0·222	0·194	0·168	0·143	0·119	0·097	0·075	0·055	0·036	0·018
5·2	1·0759	0·260	0·230	0·202	0·175	0·149	0·125	0·102	0·079	0·058	0·038	0·019
5·3	1·0018	0·265	0·235	0·207	0·180	0·154	0·129	0·105	0·082	0·061	0·040	0·019
5·4	0·9357	0·267	0·238	0·210	0·183	0·157	0·132	0·108	0·085	0·062	0·041	0·020
5·5	0·8764	0·266	0·238	0·210	0·183	0·158	0·133	0·109	0·086	0·063	0·041	0·020
5·6	0·8230	0·262	0·235	0·208	0·182	0·157	0·132	0·109	0·086	0·063	0·042	0·021
5·7	0·7749	0·256	0·229	0·203	0·178	0·154	0·130	0·107	0·085	0·063	0·041	0·020
5·8	0·7313	0·247	0·222	0·197	0·173	0·150	0·127	0·105	0·083	0·061	0·040	0·020
5·9	0·6917	0·235	0·212	0·189	0·166	0·144	0·122	0·101	0·080	0·059	0·039	0·019
6·0	0·6557	0·222	0·200	0·179	0·158	0·137	0·116	0·096	0·076	0·057	0·038	0·019
6·1	0·6227	0·208	0·188	0·168	0·148	0·129	0·109	0·091	0·072	0·054	0·035	0·018
6·2	0·5926	0·192	0·174	0·156	0·137	0·120	0·102	0·084	0·067	0·050	0·033	0·016
6·3	0·5649	0·176	0·159	0·143	0·126	0·110	0·094	0·078	0·062	0·046	0·031	0·015
6·4	0·5394	0·160	0·145	0·130	0·115	0·100	0·085	0·071	0·056	0·042	0·028	0·014
6·5	0·5158	0·143	0·130	0·116	0·103	0·090	0·077	0·064	0·051	0·038	0·025	0·013
6·6	0·4940	0·127	0·115	0·104	0·092	0·080	0·068	0·057	0·045	0·034	0·023	0·011
6·7	0·4739	0·112	0·102	0·091	0·081	0·071	0·060	0·050	0·040	0·030	0·020	0·010
6·8	0·4551	0·097	0·088	0·079	0·070	0·061	0·053	0·044	0·035	0·026	0·017	0·009
6·9	0·4376	0·084	0·076	0·068	0·061	0·053	0·045	0·038	0·030	0·023	0·015	0·008
7·0	0·4214	0·071	0·065	0·058	0·052	0·045	0·039	0·032	0·026	0·019	0·013	0·006
7·1	0·4062	0·060	0·055	0·049	0·044	0·038	0·033	0·027	0·022	0·016	0·011	0·005
7·2	0·3919	0·050	0·046	0·041	0·036	0·032	0·027	0·023	0·018	0·014	0·009	0·005
7·3	0·3786	0·041	0·038	0·034	0·030	0·026	0·023	0·019	0·015	0·011	0·007	0·004
7·4	0·3661	0·034	0·031	0·028	0·025	0·021	0·018	0·015	0·012	0·009	0·006	0·003
7·5	0·3543	0·027	0·025	0·022	0·020	0·017	0·015	0·012	0·010	0·007	0·005	0·002
7·6	0·3432	0·022	0·020	0·018	0·016	0·014	0·012	0·010	0·008	0·006	0·004	0·002
7·7	0·3327	0·017	0·016	0·014	0·013	0·011	0·009	0·008	0·006	0·005	0·003	0·002
7·8	0·3228	0·014	0·012	0·011	0·010	0·009	0·007	0·006	0·005	0·004	0·002	0·001
7·9	0·3134	0·010	0·010	0·009	0·008	0·007	0·006	0·005	0·004	0·003	0·002	0·001
8·0	0·3046	0·008	0·007	0·007	0·006	0·005	0·004	0·004	0·003	0·002	0·001	0·001
8·1	0·2962	0·006	0·006	0·005	0·004	0·004	0·003	0·003	0·002	0·002	0·001	0·001
8·2	0·2882	0·005	0·004	0·004	0·003	0·003	0·002	0·002	0·002	0·001	0·001	—
8·3	0·2806	0·003	0·003	0·003	0·002	0·002	0·002	0·002	0·001	0·001	0·001	—
8·4	0·2734	0·002	0·002	0·002	0·002	0·002	0·001	0·001	0·001	0·001	—	—
8·5	0·2666	0·002	0·002	0·001	0·001	0·001	0·001	0·001	0·001	—	—	—
8·6	0·2600	0·001	0·001	0·001	0·001	0·001	0·001	0·001	—	—	—	—
8·7	0·2538	0·001	0·001	0·001	0·001	0·001	—	—	—	—	—	—
8·8	0·2478	0·001	0·001	0·001	—	—	—	—	—	—	—	—
8·9	0·2421	—	—	—	—	—	—	—	—	—	—	—
9·0	0·2367	—	—	—	—	—	—	—	—	—	—	—

The provisional expected probit for each dose is then used to give the working probit, as ordinarily defined, and also a value of t. As proved elsewhere (Finney, 1949), b, an improved estimate of the regression coefficient, and $\delta C/(1-C)$ are the multiple regression coefficients of the working probits on x and t, each y being

assigned a weight nw. Thus, if S_{xx} denotes the weighted sum of squares of deviations $Snw\,(x-\bar{x})^2$, S_{xt} the weighted sum of products of deviations $Snw\,(x-\bar{x})\,(t-\bar{t})$, and so on,

$$\left.\begin{aligned} bS_{xx}+\frac{\delta C}{1-C}S_{xt}&=S_{xy},\\ bS_{xt}+\frac{\delta C}{1-C}S_{tt}&=S_{ty}.\end{aligned}\right\} \tag{5}$$

If, in addition to the treated insects, n_c have been kept as controls, and of these r_c die,

$$c=r_c/n_c \tag{6}$$

is an estimate of C from the controls alone. This need not itself be used as the starting-point of the calculations, if the general appearance of the data suggests that some other value is more nearly the best estimate, but allowance for the controls must be made by adding

$$\frac{n_c(1-C)}{C} \tag{7}$$

to S_{tt}, and

$$\frac{n_c(c-C)}{C} \tag{8}$$

to S_{ty}. After solution of the equations for b and $\delta C/(1-C)$, a is calculated as

$$a=\bar{y}-b\bar{x}-\frac{\delta C}{1-C}\bar{t} \tag{9}$$

(a is here defined differently from the a of the 1944 paper), and the probit regression line is written

$$Y=a+bx. \tag{10}$$

In the usual manner, the calculations may then be repeated, replacing C by $(C+\delta C)$ and using expected probits given by equation (10).

These and later stages of the calculations can best be described in terms of a numerical example. The data on the toxicity of pyrethrins to eggs of *Ephestia kühniella* used previously (1944) can conveniently be used again, and Table 2 contains the first steps. Successive columns show the log dose, the number of eggs tested (n), the number failing to hatch (r), the percentage failure (p^*) by equation (1), the percentage adjusted by equation (2) for a provisional natural inviability rate of 59% (p), and the probit of p. The expected probit (Y) is obtained from a sketch or by inspection, in the usual manner, and Table 1 is used to give the weights and t, the auxiliary variate.† The working probits (y) are found and the remainder of the

† In fact, for Table 2, w was taken from the more detailed manuscript tables, since three-figure accuracy seemed scarcely adequate for the lowest dose. For most practical purposes, interpolation in Table 1 will suffice. The simpler character of the new scheme of computation makes clear that unnecessary digits were retained in the calculations in the 1944 paper. In accordance with standard practice described elsewhere (Finney, 1947), p need be calculated only to the nearest 1%, and therefore the p^* and p columns of Table 2—given for convenience as percentages, not proportions—have been altered from the corresponding columns in the previous paper. The effect on the results of the analysis is trivial; the saving of time through the possibility of using standard tables of working probits is appreciable.

table completed by familiar processes, the only new features being the inclusion of the auxiliary variate and the extra contributions to S_{tt} and S_{tu} from the controls, formulae (7) and (8).

TABLE 2. *Analysis of results of spraying eggs of* Ephestia kühniella *with pyrethrins*

x	n	r	$p\%$	$(C=59)$ p	Empirical probit	Y	nw	t	y	nwx	nwt	nwy
1·70	50	50	100·0	100	—	7·3	1·54	0·38	7·68	2·6180	0·5852	11·8272
1·40	48	46	95·8	90	6·28	6·2	6·77	0·59	6·28	9·4780	3·9943	42·5156
1·00	51	38	74·5	38	4·69	4·7	6·59	1·62	4·70	6·5900	10·6758	30·9730
0·70	49	32	65·3	15	3·96	3·6	0·786	6·14	4·06	0·5502	4·8260	3·1912
0·40	51	32	62·7	9	3·66	2·5	0·0107	56·70	7·28	0·0043	0·6067	0·0779
Controls	99	55	55·6									
							15·6967			19·2405	20·6880	88·5849

$\bar{x}=1{\cdot}2258$ $\bar{t}=1{\cdot}3180$ $\bar{y}=5{\cdot}6435$

$Snwx^2$	$Snwxt$	$Snwt^2$	$Snwxy$	$Snwty$	$Snwy^2$
24·6967	20·8847	83·9053	112·866	103·765	516·93
23·5844	25·3587	27·2665	108·584	116·753	499·93
1·1123	−4·4740	56·6388	4·282	−12·988	17·00
	$n_c(1-C)/C=$	68·7966	$n_c(c-C)/C=$	−5·705	
		125·4354		−18·693	

The coefficients of equations (5) are read from Table 2, to give

$$1{\cdot}1123\,b-4{\cdot}4740\,\frac{\delta C}{1-C}=4{\cdot}282,$$

$$-4{\cdot}4740\,b+125{\cdot}4354\,\frac{\delta C}{1-C}=-18{\cdot}693.$$

The inverse matrix method for the solution of such equations has often been described (Finney, 1947). If Δ is defined by

$$\Delta=1{\cdot}1123\times125{\cdot}4354-(4{\cdot}4740)^2,$$

the inverse matrix is

$$V=\begin{pmatrix}\frac{125{\cdot}4354}{\Delta} & \frac{4{\cdot}4740}{\Delta}\\ \frac{4{\cdot}4740}{\Delta} & \frac{1{\cdot}1123}{\Delta}\end{pmatrix}$$

$$=\begin{pmatrix}1{\cdot}0496 & 0{\cdot}03744\\ 0{\cdot}03744 & 0{\cdot}009308\end{pmatrix}.$$

Hence $b=4{\cdot}282\times1{\cdot}0496-18{\cdot}693\times0{\cdot}03744$

$=3{\cdot}795,$

and
$$\frac{\delta C}{1-C} = 4{\cdot}282 \times 0{\cdot}03744 - 18{\cdot}693 \times 0{\cdot}009308$$
$$= -0{\cdot}0137.$$

The revised estimate of the remaining parameter, a, is obtained from equation (9), with the aid of the weighted means shown in Table 2:

$$a = 5{\cdot}6435 - 3{\cdot}795 \times 1{\cdot}2258 + 0{\cdot}0137 \times 1{\cdot}3180$$
$$= 1{\cdot}010.$$

The regression line is therefore

$$Y = 1{\cdot}010 + 3{\cdot}795x, \tag{11}$$

and the estimate of control mortality

$$C + \delta C = 0{\cdot}59 - 0{\cdot}41 \times 0{\cdot}0137$$
$$= 0{\cdot}5844.$$

Insertion of the five experimental values of x in the regression equation gives to Y values very little different from those used in Table 2, and, since δC is small, it seems unlikely that a further cycle of computation will be needed.

One inconvenience in the form of calculation originally suggested for this type of problem is that the χ^2 test for heterogeneity or deviations from the hypothesis expressed by the linear regression of probit on log dose (i.e. a normal distribution of individual log lethal doses) required evaluation of expected frequencies of death and survival for every dose. The analogy between the new calculations and those for multiple regression shows that

$$\chi^2_{[3]} = S_{yy} - bS_{xy} - \frac{\delta C}{1-C} S_{ty} \tag{12}$$
$$= 17{\cdot}00 - 3{\cdot}795 \times 4{\cdot}282 - 0{\cdot}0137 \times 18{\cdot}693$$
$$= 0{\cdot}49.$$

Since three parameters, a, b, and C, have been estimated from the data, the degrees of freedom are three less than the total number of doses (counting the untreated controls as one dose), or 3 for this example, and clearly χ^2 does not approach significance. Had χ^2 been near the borderline of significance, evaluation of expected frequencies would have been necessary in order to ensure that contributions from classes with very small expectation were not playing an undue part in the size of χ^2: this is a standard procedure with probit analysis (Finney, 1947, § 18), which fortunately is not often required. Here there is no reason to suspect that deviations from predictions based on the fitted parameters are due to anything but the chances of random sampling.

The elements of V are the variances of b and of $\delta C/(1-C)$, and the covariance of these two. Hence

$$\text{S.E.}\,(b) = \sqrt{1{\cdot}0496} = 1{\cdot}024,$$

and
$$\text{S.E.}\,(C) = 0{\cdot}41\sqrt{0{\cdot}00931} = 0{\cdot}0396,$$

where the latter now relates to the *revised* estimate, previously called $(C+\delta C)$. The changes from the estimate of C and the regression coefficient, 0·59 and 3·7 respectively, used in initiating the calculations of Table 2 are so small in comparison with these standard errors that a further cycle of computation would obviously be wasted. Had it been needed, Table 2 would have been recomputed, starting from an inviability rate of 58·44% and a set of expected probits given by equation (11).

The log LD 50 is given by

$$m=(5-a)/b \tag{13}$$
$$=3\cdot990/3\cdot795$$
$$=1\cdot051.$$

The approximate formula for the variance of m is

$$V(m)=\frac{1}{b^2}\left[\frac{1}{Snw}+\bar{t}^2V\left(\frac{C}{1-C}\right)-2(m-\bar{x})\bar{t}\ \mathrm{Cov}\left\{b,\frac{C}{1-C}\right\}+(m-\bar{x})^2\,V(b)\right] \tag{14}$$
$$=[0\cdot0637+(1\cdot318)^2\times0\cdot00931+2\times0\cdot175\times1\cdot318\times0\cdot0374+(0\cdot175)^2\times1\cdot0496]\div(3\cdot795)^2$$
$$=[0\cdot0637+0\cdot0162+0\cdot0173+0\cdot0321]\div14\cdot402$$
$$=0\cdot00898.$$

The standard error obtained as the square root of this, 0·095, cannot rightly be used in assigning 5% fiducial limits to m, however, since

$$g=t^2V(b)/b^2$$
$$=3\cdot84\times1\cdot050\div14\cdot40$$
$$=0\cdot28;$$

here t is the 5% normal deviate (1·96), and the size of g indicates that the exact formula for fiducial limits must be used (Finney, 1947, § 19). The formula required is numbered (4·7) in the book just quoted; written in terms of the present notation, it becomes

$$m+\frac{g}{1-g}\left[m-\bar{x}-\frac{\bar{t}\ \mathrm{Cov}\left\{b,\frac{\delta C}{1-C}\right\}}{V(b)}\right]$$
$$\pm\frac{1\cdot96}{b(1-g)}\sqrt{\left[\frac{1}{Snw}+\bar{t}^2V\left(\frac{\delta C}{1-C}\right)-2(m-\bar{x})\bar{t}\ \mathrm{Cov}\left\{b,\frac{\delta C}{1-C}\right\}+(m-\bar{x})^2\,V(b)\right.}$$
$$\left.-g\left\{\frac{1}{Snw}+\bar{t}^2\,V\left(\frac{\delta C}{1-C}\right)-\frac{\bar{t}^2\ \mathrm{Cov}^2\left\{b,\frac{\delta C}{1-C}\right\}}{V(b)}\right\}\right], \tag{15}$$

where 1·96 is inserted for the 5% normal deviate in order to avoid confusion with the auxiliary variate, t. The arithmetical values derived as the fiducial limits to m are 1·200 and 0·730. Since x was the logarithm of $10^3\times$ percentage concentration of

pyrethrins, the antilogarithms of m and its limits give 0·0112% as the estimated LD 50, with 5% limits at 0·0158 and 0·0054%.

Apart from minor differences introduced by rounding off of superfluous digits at different stages, the calculations in this paper are equivalent to those in the 1944 paper, and all numerical values are essentially the same. The earlier paper was at fault, however, in quoting a simple standard error for the LD 50, as, with these data, the exact procedure for fiducial limits is necessary.

REFERENCES

FINNEY, D. J. (1944). The application of the probit method to toxicity test data adjusted for mortality in the controls. *Ann. appl. Biol.* **31**, 68–74.

FINNEY, D. J. (1947). *Probit Analysis: A Statistical Treatment of the Sigmoid Dose Response Curve.* London: Cambridge University Press.

FINNEY, D. J. (1949). The estimation of the parameters of tolerance distributions. *Biometrika*, **36**. (In the press.)

FISHER, R. A. & YATES, F. (1948). *Statistical Tables for Biological, Agricultural and Medical Research*, 3rd ed. Edinburgh: Oliver and Boyd.

HORSFALL, J. G. (1945). *Fungicides and Their Action.* Waltham, Mass., U.S.A.: Chronica Botanica Co.

(*Received 27 October* 1948)

DOSAGE-MORTALITY CORRELATION WITH NUMBER TREATED ESTIMATED FROM A PARALLEL SAMPLE*

By F. M. WADLEY

U.S. Department of Agricuture, Washington, D.C.

When the number of organisms treated in a dosage-mortality study is not known exactly, but is estimated from a sample, weighting must be modified. Weights based on Poisson expectation are worked out and tabled. Maximum likelihood equations, designed to improve the estimate of number treated, are presented. Two numerical examples are worked out to illustrate the use of the weights and equations.

The problem

In some dosage-mortality studies, experimental conditions are such that the number of organisms treated is not known exactly, but must be estimated from an untreated sample. This has been observed recently in bacteriological studies. A sample of the medium gives an estimate of the population per unit volume. Other similar samples are treated and survivors are counted. Another example is found in treatment of fruit infested with immature stages of fruit-flies (Baker, 1939). Samples of fruit are given treatments of graded severity, and survivors are counted. The original population is estimated by rearing a similar sample or samples. In such problems, exact counts of living and dead would either be impracticable, or would be so laborious as to limit sharply the total number used.

The procedure has led to some special questions in dosage-mortality correlation. Regression techniques developed by Bliss and Fisher (Bliss 1935, 1938), and elaborated by Finney (1947), have given fairly good results in such problems. However, weighting must obviously be modified when the original population is not known exactly.

It is of interest to note that the studies have in some cases been of temperature treatments. The treatments have consisted of varying time of exposure to an unfavourable temperature. A moderately high or moderately low temperature continued for some time will kill fruit-fly larvae without injury to the fruit. High-temperature treatments are often used against bacteria and other organisms. Results have been treated by methods of probit analysis, using time of exposure as dosage. The curves seem more closely related to Bliss's time-mortality curves (1937) than to typical dosage-mortality curves. In some cases, especially with fruit-flies, better results are given by using untransformed time units than by using their

* The kind encouragement of Prof. R. A. Fisher and Prof. W. G. Cochran, and the helpful advice of Mr D. J. Finney, must be acknowledged. Mr Finney supplied some values of special accuracy for Table 1.

logarithms. Problems of regression and of weighting are not altered by change of scale of the independent variable.

Weighting

Weights used in such problems are essentially the reciprocals of estimated variances of the probit values. If the probits are from estimated rather than from counted populations, variances are obviously higher. The added uncertainty as to the exact proportion affected should be reflected in the variance and the weight.

If n, the number of organisms treated, is estimated from a parallel sample, while s, the number surviving, is known exactly, the proportion surviving, q, will be estimated as s/n. This estimate will have two sources of variance. One is the familiar binomial variance, pq/n, which would be present even if n were known exactly. The other is the additional variance caused by uncertainty about the value of n. The two should be independent and additive.

Table 1. *Weighting coefficients for the case where n is known only from a parallel sample, assuming Poisson variation*

Mortality estimated (probits)	Weighting coefficient (z^2/q)	Mortality estimated (probits)	Weighting coefficient (z^2/q)	Mortality estimated (probits)	Weighting coefficient (z^2/q)
3·5	0·0180	5·0	0·3183	6·5	0·2511
3·6	0·0244	5·1	0·3424	6·6	0·2245
3·7	0·0325	5·2	0·3634	6·7	0·1985
3·8	0·0426	5·3	0·3807	6·8	0·1735
3·9	0·0549	5·4	0·3936	6·9	0·1499
4·0	0·0696	5·5	0·4017	7·0	0·1281
4·1	0·0868	5·6	0·4049	7·1	0·1083
4·2	0·1065	5·7	0·4030	7·2	0·0905
4·3	0·1286	5·8	0·3961	7·3	0·0748
4·4	0·1530	5·9	0·3847	7·4	0·0612
4·5	0·1793	6·0	0·3690	7·5	0·0495
4·6	0·2069	6·1	0·3498	7·6	0·0396
4·7	0·2354	6·2	0·3277	7·7	0·0313
4·8	0·2640	6·3	0·3034	7·8	0·0245
4·9	0·2919	6·4	0·2776	7·9	0·0190
				8·0	0·0146

The error of estimating n treated from the untreated sample might be considered as containing two parts: the error of n in various treated samples about the mean value, and the error of estimating the mean from the untreated lots. The latter affects all samples alike and may not be very important if untreated material is ample. The variance of n about the mean under simple sampling conditions, is that of the Poisson, simply n. This may be used as a basis for weights. Under practical conditions the variance will often exceed this value, but it may be regarded as a useful approximation and a lower limit. For some cases the negative binomial might be used (see discussion after Table 5).

Since $q = s/n$ or $s.n^{-1}$; $dq/dn = -s/n^2$. Variance of p or q arising from variable n is

estimated as $(dq/dn)^2 \times$ (variance n); or $(s^2/n^4).n = s^2/n^3 = q^2/n$. The binomial variance, pq/n, may be written $(q-q^2)/n$. The sum of the two components is simply q/n, the expected variance of a proportion q (or p) under such conditions. Following Bliss (1935), the variance of a probit of such a value is q/nz^2, where z is the ordinate of the normal curve. The reciprocal of the variance, the weight, is nz^2/q; the weighting coefficient (w), which may be tabled, is z^2/q.

Weighting coefficients calculated as z^2/q differ in several ways from standard coefficients. It is quite noticeable that they are not symmetrical about 50%. The maximum value is at almost 5·6 probits, over 70%. At low estimated mortalities, the weight is very low. This would be expected; if original number is uncertain and survivors numerous it is uncertain whether any at all have been killed.

These weighting coefficients are tabled (Table 1) for probit values by tenths from 3·5 to 8·0. Below 3·5 the weights are low and mortalities of small interest. Above 8·0, weights are so close to Bliss's that the differences are unimportant.

Maximum likelihood estimates

In many cases the standard procedure (Bliss, 1935, 1937, 1938; Finney, 1944, 1947) with the modified weighting will give good results. In some cases, however (especially when low mortality treatments are included), the estimate of n may be improved a little. In such cases the data may be studied and equations solved for adjustment to n, as well as for the regression constants a and b. (These constants are from the regression equation $Y = a + bX$, where Y is a probit value and X dosage.)

In deriving these equations it may be stated that if s is the number of survivors, m the average number treated, n an estimate of m, q the survival rate, and Poisson distribution is assumed:

Probability of n in an untreated sample is $e^{-m}.m^n/n!$,

Probability of s in a treated sample is $e^{-mq}.(mq)^s/s!$.

The logarithm of likelihood for a typical series of treatments is

$$L = \text{constant} - m + n.\log m - m.S(q) + \log m.S(s) + S(s.\log q),$$

where S indicates summation.

Partial derivatives of L with respect to m, a and b are readily derived. The latter two are derived through their relation to q: $dq/da = z$, $dq/db = zx$, where normality is assumed. Second partial derivatives and cross partials may also be derived, and available estimates substituted for theoretical values. Further development follows a path similar to Finney's (1947, Appendix II); three equations similar to his set II(3) are set up to give δN, δa, δb. These are adjustments to give improved values of m, a, and b, where preliminary estimates are used to begin with. With the improved values a second set of calculations may be made, and so on until adjustments become negligible.

The final equations are produced by simplifying the three equations mentioned, still following Finney's procedure (1947). The regression equation is modified to

$a'+b\,(x-\bar{x})$, where $a'=a+b\bar{x}$. The adjustment equations are likewise modified to give estimates of a', b and δN; the working probit (Bliss, 1935, 1937, 1938; Finney, 1944, 1947) is introduced, making substitution of $y-Y$ for $(Q-q)/z$. Here Y and Q are values estimated from the provisional line. The quantity z^2/Q is replaced by w; N is used for the estimate of m, n for the sample value. The latter two are identical in the first approximation. The equations are then as follows:*

$$[1+S\,(Q)]/N.\delta N \quad -S\,(z).a' \quad -S\,[z\,(x-\bar{x})].b \quad =n/N-1-S\,(zy), \tag{1}$$

$$-S\,(z).\delta N \quad +S\,(Nw).a' \quad =S\,(Nwy), \tag{2}$$

$$-S\,[z\,(x-\bar{x})].\delta N \quad +S\,[Nw\,(x-\bar{x})^2].b \quad =S\,[Nw\,(x-\bar{x})\,(y-\bar{y})]. \tag{3}$$

These are easily solved if the needed totals are calculated and used as coefficients. It will be observed that if δN is negligible, equations (2) and (3) give simple weighted regression estimates of a' (or $\bar{y}$) and b. If the new values are used, calculations can be repeated, until estimates of a', b and n are changing but little. Calculations of variance, χ^2, interpretation, etc., can be made following Finney (1947, ch. 6).

Numerical examples

The first example, partly hypothetical but closely corresponding to actual data, is drawn from material furnished by Dr Reynolds of the Bureau of Human Nutrition and Home Economics, U.S. Department of Agriculture. Spore suspensions of a bacterial species affecting food were held at 107° C. for several time periods. Counts of survivors per unit volume were made on each; also on an untreated sample from the same culture. The sample estimate showed 280 per unit. On plotting it was found that this particular lot gave somewhat better linearity when probits of estimated mortality were plotted against logs of time units, than when untransformed time values were used. The reverse is true in some of this material. In this case logs were used; a preliminary graphic estimate of the regression was:

$$\text{Probit mortality} = -6{\cdot}72+8{\cdot}20\ (\text{log no. minutes}).$$

Data are shown in Table 2 and calculations proceeded as in Table 3.

Figures are set up for hand calculation; machine work will permit some short cuts. The totals yield values for substitution in the equations; $[1+S\,(Q)]/N$ is

* Mr Finney suggests the following more general form of the equations for k untreated samples:

$$\delta N.[kN+S\,(Nw\,Q^2/z^2)]/N^2-a'/N.S\,(Nw\,Q/z)-b/N.S\,[Nw\,(x-\bar{x})\,Q/z] = k\,(\bar{n}/N-1)-1/N\,[S\,(Nwy\,Q/z)], \tag{1}$$

$$-\delta N/N.S\,(Nw\,Q/z) \quad +a'.S\,(Nw) \quad =S\,(Nwy), \tag{2}$$

$$-\delta N/N.S\,[Nw\,(x-\bar{x})\,Q/z] \quad +b.S\,[Nw\,(x-\bar{x})^2]=S\,[Nw\,(x-\bar{x})\,(y-\bar{y})]. \tag{3}$$

This will be found to be identical with the equations given above when $\bar{n}$ is used as the estimate of N, and $k=1$, since $w=Z^2/Q$. Finney's equations show well the relations involved, and the analogy to his equations considering natural mortality (1944).

2·286/280 or 0·0081643. Then $\bar{x}$ and $\bar{y}$ are estimated as $S(wx)/S(w)$, $S(wy)/S(w)$. $S[w(x-\bar{x})^2]$ is estimated as $S(wx^2)-\bar{x}.S(wx)$; $S[w(x-\bar{x})(y-\bar{y})]$ as

$$S(wxy)-\bar{x}.S(wy).$$

Several of the totals are multiplied by N as indicated in the equations; $n/N-1-S(zy)$ is simply $-S(zy)$ in the first approximation, while in later trials n/N becomes the original estimate of n divided by the latest one.

TABLE 2. *Data on bacterial mortality*

No. min.	Log min. (X)	s	q (s/280)	p	Empirical probit	Expected probit (Y) ($-6·72+8·2X$)
20	1·30	233	0·832	0·168	4·04	3·94
30	1·48	85	0·304	0·696	5·51	5·42
40	1·60	29	0·104	0·896	6·26	6·40
50	1·70	3	0·011	0·989	7·29	7·22
			1·251			

TABLE 3. *First approximation, data of Table 2*

X	Q exp.	Z	(z^2/Q)	Working probit (y)	wy	wx	wx^2	wxy	$x-\bar{x}$	$z(x-\bar{x})$	zy
1·30	0·855	0·228	0·061	4·04	0·2464	0·0793	0·1031	0·3203	−0·231	−0·052668	0·92112
1·48	0·337	0·365	0·395	5·51	2·1764	0·5846	0·8652	3·2211	−0·051	−0·018615	2·01115
1·60	0·081	0·150	0·278	6·25	1·7375	0·4448	0·7117	2·7800	+0·069	+0·010350	0·93750
1·70	0·013	0·034	0·089	7·28	0·6479	0·1513	0·2572	1·1014	+0·169	+0·005746	0·24752
Total	1·286	0·777	0·823	—	4·8082	1·2600	1·9372	7·4228	—	−0·055187	4·11729

TABLE 4. *Successive approximations*

Values after	δN	N	a'	a	b
Start	—	280	—	−6·72	8·20
1st approx.	+0·9	280·9	5·85	−5·73	7·56
2nd approx.	+0·4	281·3	5·83	−5·74	7·57
3rd approx.	−0·1	281·2	5·81	−5·80	7·61
4th approx. (partial)	—	281·2	5·82	−5·76	7·58

Equations are then as follows:

$$+0·0081643\,\delta N-0·777\,a'+0·055187\,b=-4·11729$$
$$-0·777\,\delta N\quad+230·44\,a'\quad=+1346·30$$
$$+0·055187\,\delta N\quad+2·268\,b\quad=+17·192.$$

Solution yields the values; $b=+7·5576$, $a'=5·8454$, $a=-5·73$,

$$\delta N=+0·9,\quad N_2=280·9.$$

Recalculation is carried out in the form of Table 3 using these new values, and another approximation is secured. In the third approximation, N becomes stable, and a fourth partial approximation is carried out for a' and b. Changes through these approximations are shown in Table 4.

For expected and actual survivals χ^2 was a little less than 2, not significant with 1 degree of freedom. Standard errors for a' and b, calculated by the inverse matrix method, were 0·083 and 0·69 respectively. The adjustment to N proved unimportant here. If it is not desired to consider N, a solution may be made for a' and b from the second and third equations; in this case values thus estimated were 5·8 and 7·58 respectively. They may be improved by further approximations.

The same mortalities, using regression on untransformed time units, gave as a final equation $Y = 2{\cdot}41 + 0{\cdot}099\,X$. χ^2 was significant, 5·9 with 1 degree of freedom. In this case the final estimate of N was 289. A somewhat higher average value of N than the sample value was evidently better suited to assumptions involved.

Another example was worked out on the fruit-fly material, using assumed data very close to actual cases from the older reports. Cold was the factor bringing about mortality, and exposure was stated in days. In this case untransformed time values were definitely better than logs, and were used. The estimated number treated was 1070; the graphic estimates of a and b were 4·13 and 0·59.

TABLE 5. *Fruit fly mortality data*

No. days (X)	Survival	q	p	Empirical probit	Expected probit
1	715	0·668	0·332	4·57	4·72
2	396	0·370	0·630	5·33	5·31
3	158	0·148	0·852	6·04	5·90
4	55	0·051	0·949	6·64	6·49
5	32	0·030	0·970	6·88	7·08
6	4	0·004	0·996	7·65	7·67

The final values were: $N = 1091$, $a' = 5{\cdot}79$, $a = 4{\cdot}12$, $b = 0{\cdot}605$. χ^2 was over 24, highly significant with 3 degrees of freedom. This indicates unsuited assumptions or heterogeneity in the material; from other evidence it is believed the latter is at least partly responsible. Standard errors for a' and b were about 0·04 and 0·02 respectively; they should be multiplied by about 3 because of the high χ^2.

Undoubtedly χ^2 is built up by the large numbers used, and more recent work of this sort shows less heterogeneity than that cited because of careful sampling. Still, such sampling of infested fruits can scarcely be expected to conform to the Poisson. The negative binomial (Fisher, 1941) might be utilized, following a development similar to that used here, and only a little more complex. It would be necessary to use several check lots. This possibility is to be explored, and may be the subject of a future article.

REFERENCES

BAKER, A. C. (1939). The basis for treatment of products where fruitflies are involved, as a condition for entry into the United States. *Circ. U.S. Dep. Agric.* no. 551.

BLISS, C. I. (1935). The calculation of the dosage-mortality curve. *Ann. appl. Biol.* **22**, 134–67.

BLISS, C. I. (1937). The calculation of the time-mortality curve. *Ann. appl. Biol.* **24**, 815–52.

BLISS, C. I. (1938). The determination of the dosage-mortality curve from small numbers. *Quart. J. Pharm.* **11**, 192–216.

FINNEY, D. J. (1944). The application of the probit method to toxicity test data adjusted for mortality in the controls. *Ann. appl. Biol.* **31**, 68–74.

FINNEY, D. J. (1947). *Probit Analysis.* Pp. 256. Cambridge University Press.

FISHER, R. A. (1941). The negative binomial distribution. *Ann. Eugen.* **11**, 182.

(*Received* 31 *July* 1948)

NOTE ON A PROBLEM IN PROBIT ANALYSIS

By F. J. ANSCOMBE

Lecturer in Mathematics, University of Cambridge

The statistical treatment of dosage-mortality data when the number of survivors is counted but not the total number of organisms in each sample, the latter being estimated from an untreated sample, has been discussed by Wadley (1949), on the assumption of Poisson variation in the number of organisms per sample. The procedure to be followed when a wider hypothesis is made that the number of organisms has a negative binomial distribution is described here; both the arithmetical analysis of the results and the optimum arrangement of the samples are considered.

In the preceding paper, F. M. Wadley discusses the statistical treatment, by probit analysis, of toxicological data where at each dosage the number s of organisms surviving is counted, but not the total number n of organisms treated, about which we only know that it is a Poisson variable with mean m. Wadley shows that s is distributed in a Poisson distribution with mean Qm, where Q is the expected proportion of survivors at the dosage in question, and that the usual technique of probit analysis can be followed, as described in Finney's book (1947), with the following modifications: (i) the 'observed percentage kill' p is calculated as $1-s/m$ (and this is used even if it should turn out to be negative, as it might at a low dosage); and (ii) in the formulae given by Finney, n is everywhere replaced by m, and the weight coefficient w is calculated by a new formula,

$$w=\frac{Z^2}{Q}. \tag{1}$$

Actually, m is not given certainly, but has to be estimated from an untreated sample, so that an estimation error in m affects all calculated probits and weights. The situation is similar to the case discussed by Finney of an estimation error in natural mortality, and it can be dealt with by maximum likelihood in the same way. It will be wise to examine a considerably larger untreated sample than any treated sample, so that this common estimation error will be small.

This theory based on a Poisson distribution for n is appropriate when samples are drawn from a well-stirred fluid in which the organisms are suspended and do not tend to aggregate or repel one another; and Wadley's first example, of spore suspensions, seems well fitted. But we should not expect to find a Poisson distribution when sampling infested fruit, and in fact Wadley's example of such material gives a very high χ^2, when treated by his method. We might then suppose that n has a negative binomial distribution with mean m and exponent k, so that the variance of n is $m+m^2/k$. It is easy to show that now s has a negative binomial distribution with

mean Qm and exponent k. Assuming to begin with that both m and k are known, the probit analysis is modified just as described above, but with weight coefficient

$$w = \frac{kZ^2}{Q\,(Qm+k)}. \tag{2}$$

In the limit as $k \to \infty$ we get Wadley's weight coefficient (1). In general, the weight coefficient (2) is more skew than (1), and is roughly equal to a fraction $k/(m+k)$ of (1) when the expected kill P is small and equal to (1) when P is large.

As before, m and k will in fact have to be estimated, with the aid of untreated samples, and there will be a common estimation error affecting all calculated probits and weights. An error in k affects the weights and estimates of standard errors, while an error in m causes in addition a bias in the fitted regression line itself.

The most obvious arrangement of the experiment is to test an equal bulk of material at each dosage and also observe several untreated samples of the same size, say u of them. If there are t dosages, the whole material available for the experiment is mixed up as well as possible and divided into $t+u$ equal lots, of which t lots selected at random are assigned to the treatments, and the remainder are untreated. The values of m and k are estimated from the u untreated lots: m is estimated as the total number of organisms in the untreated lots divided by u, and there are several possible methods of estimating k (Fisher, 1941; Anscombe, 1949). Unless u is fairly large, the estimation error in m and k, particularly the latter, will be large. Without otherwise affecting the efficiency of the procedure, the following device, whenever it is practicable, will enable k to be estimated more accurately.

The material to be used in the experiment is first well mixed (this can certainly be done with fruit, though perhaps not very conveniently). It is then divided into $r\,(t+u)$ equal lots, where r may be 2 or 3 or any larger integer, r of the lots selected at random are assigned to each treatment, and the remaining ru lots are untreated. If the mixing was adequate, the number of organisms per sample will have a negative binomial distribution with mean and exponent equal to one-rth their previous values (i.e. one-rth the values they would have had for $r=1$). The observations from the sample are pooled together in the sets of r at each dosage and the calculations go just as before, except for the estimation of k. The value of k can be estimated from the set of ru values of n in the untreated samples and also from the sets of r values of s at each dosage, as these variables all have negative binomial distributions with the same exponent. The larger r is, the better, in principle (i.e. the accuracy of estimation of k is higher), but in practice it will be unwise to take r very large, since imperfect mixing of the material or a departure from a strict negative binomial form of distribution may lead to error. In fact, if r is large, the exponent for individual samples will be small, and will be conveniently estimated from the proportion of uninfested samples. The value of k so obtained will then be used in the probit analysis to give the variance of the observations. We are thus relying on the proportion of zeros in

individual samples being related to the variance of the total number of survivors in sets of r samples as the negative binomial theory predicts.

The reasonableness of the assumption of a negative binomial distribution could be checked up from the data themselves, in such an experiment; and it would be possible to obtain a fair analysis without making any assumption about the distribution of n or s, provided r was not too small. The observed variance of s at each dosage would be plotted against its average value and a smooth curve fitted.

REFERENCES

Anscombe, F. J. (1949). The statistical analysis of insect counts, based on the negative binomial distribution. *Biometrics*. (In the press.)

Finney, D. J. (1947). *Probit Analysis*. Cambridge University Press.

Fisher, R. A. (1941). The negative binomial distribution. *Ann. Eugen.* **11**, 182.

Wadley, F. M. (1949). Dosage-mortality correlation with number treated estimated from a parallel sample. *Ann. appl. Biol.* **36**, 196.

(*Received* 11 *November* 1948)

THE INSECTICIDAL ACTION OF SOME D.D.T. ANALOGUES AND CHLORINATED (4-CHLOROPHENYL)-ETHANES

By A. STRINGER

Long Ashton Research Station, Bristol

The insecticidal effectiveness of D.D.T. is dependent upon the combination of the two chlorophenyl groups with the trichloroethane group. Modification of the latter group results in a loss of toxicity. The toxicity of the compounds possessing $=CH.CCl_3$, $=CH.CHCl_2$ and $=CH.CH_2Cl$ groups decreases as the degree of chlorination decreases.

None of the chlorinated (4-chlorophenyl)-ethane compounds are of the same order of toxicity as D.D.T. The relative potency increases with increasing chlorine content of the side-chain, although complete chlorination results in a compound possessing negligible toxicity. A relationship between toxicity and chlorine content is also seen in the ethylenic compounds, which were formed as intermediates in the synthesis of the ethane derivatives. The comparatively non-toxic ethylenic derivatives of D.D.T. when contrasted with $(4\text{-}ClC_6H_4)\ CCl=CCl_2$, suggests that the spatial configuration of the molecule may be of importance.

It has been the aim of many workers to demonstrate some law correlating the insecticidal action of organic compounds with their chemical structure. Although the precise mode of action of D.D.T. has not been determined, its insecticidal activity has been attributed to the presence of two (4-chlorophenyl) groups (Laüger, Martin & Müller, 1944) and also to the loss of hydrogen chloride from the $=CH.CCl_3$ group (Martin & Wain, 1944*b*). On the other hand, it has been suggested that such explanations of the biological activity of D.D.T. are too simple and direct, and it is probable that the molecular configuration is also of importance (Busvine, 1945).

The active ingredient of a German insecticide, formerly called 'Lucex' and prepared by the side-chlorination of 4-chloro-1-ethyl benzene, contains one 4-chlorophenyl group and a side-chain, whose precise structure is not known but is said to be chlorinated to about $—C_2HCl_4$ (Martin & Shaw, 1946). Some of the compounds likely to be present in 'Lucex' were prepared by Dr D. Woodcock (1948) and their relative toxicities to *Calandra granaria* L. were investigated. The D.D.T. analogues examined were prepared by Dr R. L. Wain (Martin & Wain, 1944*a*).

Technique

The test insects were adult grain weevils (*Calandra granaria* L.). The usual precautions were taken to standardize the rearing and pretreatment of the insects and the method of testing. Batches of 50 *Calandra* adults were enclosed, by means of glass rings, on filter-papers which had been previously treated with insecticide (Stringer, 1948). Deposits from acetone solutions were used with the 4-chlorophenyl-ethane series

and oil-films with the D.D.T. analogues. The results were analysed by the usual statistical procedure, the regression of probit mortality on log dosage (Finney, 1947).

Results

(i) *The effects of p-substituents on the toxicity of D.D.T.*

Previous work on the qualitative examination of the insecticidal activity of D.D.T. analogues (Martin, Stringer & Wain, 1943 and Martin & Wain, 1944*a*) suggested that the insecticidal properties were to some extent associated with the *p*-chloro-derivative, and that with the examination of *p*-substituents it was concluded that the chlorophenyl groups conferred high lipoid solubility and hence high permeativity.

Table 1 gives the results of tests of *p*-bromo- and *p*-iodo-derivatives against D.D.T., with the concentration measured in 10^{-6} moles per ml. It is seen that the departure from parallelism in both cases is not significant and the relative potencies indicate that the chloro- and bromo-compounds are equitoxic and the iodo-compound is less than half as toxic as D.D.T.

(ii) *The effect on toxicity of modifications in the trichloroethylidene group of D.D.T.*

In addition to D.D.T. the following compounds were tested:

αα-bis(4-chlorophenyl)-ethane.

αα-bis(4-chlorophenyl)-β-chloroethane.

αα-bis(4-chlorophenyl)-ββ-dichloroethane.

αα-bis(4-chlorophenyl)-αβ-dichloroethane.

αα-bis(4-chlorophenyl)-αβββ-tetrachloroethane.

αα-bis(4-chlorophenyl)-ethylene.

αα-bis(4-chlorophenyl)-β-chloroethylene.

αα-bis(4-chlorophenyl)-ββ-dichloroethylene.

With the exception of αα-bis(4-chlorophenyl)-ββ-dichloroethane (D.D.D.), these compounds possessed little or no toxic action against *Calandra*, and only αα-bis(4-chlorophenyl)-ethane gave kills which could be compared with those resulting from D.D.T. The following regression lines were obtained:

$$(1)\quad y = 2{\cdot}575\,x + 4{\cdot}358 \quad \text{for} \quad \text{D.D.T.},$$

and

$$(2)\quad y = 2{\cdot}575\,x - 1{\cdot}566 \quad \text{for} \quad (ClC_6H_4)_2CHCH_3;$$

$$x = \log(\text{moles}/10^6\ \text{ml}).$$

χ^2 for goodness of fit was 13·346 for 10 D.F. and $M_{12} = -2{\cdot}3007 \pm 0{\cdot}0609$. Thus the relative potency of $(ClC_6H_4)_2CH.CH_3$ with respect to D.D.T. was 0·005. αα-bis(4-chlorophenyl)-ββ-dichloroethane (D.D.D.) was tested over a concentration range of

TABLE 1. *Toxicity of p-bromo-, and p-iodo-substituents of D.D.T. to* Calandra granaria

	Equation	b	m	M	Relative potency	χ^2
(*a*) $(p\text{-Cl.C}_6\text{H}_4)_2\text{CHCCl}_3$	$y=1{\cdot}470x+4{\cdot}081$	$1{\cdot}470\pm0{\cdot}190$	$0{\cdot}625\pm0{\cdot}054$	—	1·00	0·572
$(p\text{-Br.C}_6\text{H}_4)_2\text{CHCCl}_3$	$y=1{\cdot}470x+4{\cdot}096$		$0{\cdot}615\pm0{\cdot}054$	$0{\cdot}0102\pm0{\cdot}0689$	1·02	$n=2$
(*b*) $(p\text{-Cl.C}_6\text{H}_4)_2\text{CH.CCl}_3$	$y=1{\cdot}445x+4{\cdot}650$	$1{\cdot}445\pm0{\cdot}206$	$0{\cdot}242\pm0{\cdot}073$	—	1·00	0·172
$(p\text{-I.C}_6\text{H}_4)_2\text{CH.CCl}_3$	$y=1{\cdot}445x+4{\cdot}077$		$0{\cdot}639\pm0{\cdot}058$	$-0{\cdot}3966\pm0{\cdot}0932$	0·40	$n=2$

Exposure time (*a*) 120 hr., (*b*) 144 hr. $x=\log$ (moles/10^6 ml.).

TABLE 2. (4-*chlorophenyl*)-*ethane derivatives. Dosage-mortality data*

Test	Compound	Equation	m	ED 50 moles $\times 10^{-6}$	Relative potencies at ED 50
a	$R.\text{CCl:CCl}_2$	$y=3{\cdot}86x+0{\cdot}68$	1·12	13·18	1·00
	$R.\text{CCl:CH}_2$	$y=2{\cdot}18x+0{\cdot}64$	2·00	100·0	0·13
b	$R.\text{CHCl.CCl}_3$	$y=2{\cdot}94x+2{\cdot}79$	0·75	5·62	1·00
	$R.\text{CHCl.CHCl}_2$	$y=4{\cdot}02x+0{\cdot}78$	1·05	11·22	0·51
	$R.\text{CHCl.CH}_2\text{Cl}$	$y=2{\cdot}80x+0{\cdot}66$	1·55	35·48	0·16
	$R.\text{CHCl.CH}_3$	$y=4{\cdot}08x-3{\cdot}28$	2·03	107·15	0·05
c	$R.\text{CCl:CH}_2$	$y=2{\cdot}34x-0{\cdot}20$	2·19	154·9	1·00
	$R.\text{CH:CH}_2$	$y=3{\cdot}96x-3{\cdot}70$	2·20	158·5	0·98
d	$R.\text{CCl}_2.\text{CCl}_3$	$y=1{\cdot}76x+1{\cdot}50$	1·99	97·7	0·03
	$R.\text{CCl}_2.\text{CHCl}_2$	$y=2{\cdot}15x+3{\cdot}86$	0·53	3·39	1·00
e	$R.\text{CCl}_2.\text{CHCl}_2$	$y=2{\cdot}99x+3{\cdot}66$	0·55	3·55	1·00
	$R.\text{CHCl.CHCl}_2$	$y=2{\cdot}91x+1{\cdot}95$	1·05	11·2	0·32
	$R.\text{CCl:CHCl}$	$y=2{\cdot}31x+1{\cdot}81$	1·38	24·0	0·15
f	$R.\text{CCl}_2.\text{CHCl}_2$	$y=3{\cdot}23x+3{\cdot}32$	0·52	3·31	1·00
	$R.\text{CHCl.CCl}_3$	$y=3{\cdot}37x+2{\cdot}14$	0·85	7·08	0·47
	$R.\text{CHCl.CHCl}_2$	$y=3{\cdot}08x+2{\cdot}01$	0·97	9·33	0·35
	$R.\text{CCl:CCl}_2$	$y=3{\cdot}27x+1{\cdot}76$	0·99	9·77	0·34
	$R.\text{CCl:CHCl}$	$y=3{\cdot}25x+0{\cdot}20$	1·48	30·2	0·11
g	$R.\text{CCl}_2.\text{CHCl}_2$	$y=2{\cdot}07x+3{\cdot}51$	0·72	5·25	1·00
	$R.\text{CCl}_2.\text{CH}_2\text{Cl}$	$y=2{\cdot}64x+0{\cdot}91$	1·55	35·48	0·15

$R=(4\text{-ClC}_6\text{H}_4)$—

Test insect *Calandra granaria*.

3·13 to $31{\cdot}26 \times 10^{-6}$ moles/ml. against D.D.T., at a concentration range 1·41 to $11{\cdot}29 \times 10^{-6}$ moles/ml., using a dosage of 0·12 ml. oil solution per filter-paper. The resultant regression lines were:

$$y = 1{\cdot}046x + 4{\cdot}314, \quad b = 1{\cdot}046 \pm 0{\cdot}244 \quad \text{for D.D.D.,}$$

and

$$y = 2{\cdot}421x + 3{\cdot}968, \quad b = 2{\cdot}421 \pm 0{\cdot}299, \quad \text{for D.D.T.}$$

The two regression lines are not parallel and hence their toxicities cannot be compared as relative potencies.

For D.D.D., $m_{50} = 0{\cdot}6558 \pm 0{\cdot}1192$ and $m_{95} = 2{\cdot}2282 \pm 0{\cdot}2909$,

and D.D.T., $m_{50} = 0{\cdot}4263 \pm 0{\cdot}0404$ and $m_{95} = 1{\cdot}1057 \pm 0{\cdot}0694$.

Thus the concentrations of D.D.D. producing 50 and 95% kill are 4·53 and $169{\cdot}1 \times 10^{-6}$ moles/ml. and for D.D.T. the corresponding concentrations are 2·67 and $12{\cdot}76 \times 10^{-6}$ moles/ml. (exposure time = 120 hr.).

(iii) *The comparative toxicities of some chlorinated (4-chlorophenyl)-ethanes*

To determine the order of toxicity of this group of compounds, the most toxic, $(4\text{-}ClC_6H_4).CCl_2.CHCl_2$, was assayed against D.D.T. Deposits of 0·12 ml. of P31 oil solutions were used at concentrations of 10 to 0·59 mg./ml. for D.D.T. and 100 to 5·9 mg./ml. for $(4\text{-}ClC_6H_4).CCl_2.CHCl_2$. Not unexpectedly, these two compounds resulted in regression lines of different slope, being respectively

$$y = 2{\cdot}05x + 2{\cdot}91,$$

$$y = 2{\cdot}87x - 2{\cdot}98;$$

x = log (ml./10 ml.). The compound $(4\text{-}ClC_6H_4).CCl_2.CHCl_2$ has the steeper slope and thus a narrower log tolerance distribution. The ED 50's and ED 95's for D.D.T. and this compound are respectively 1·05 and 6·61 mg./ml. as compared with 60·3 and 223·9 mg./ml. Thus for the ED 50 level of mortality D.D.T. is 58 times as toxic and for the ED 95 D.D.T. is 34 times as toxic as $(4\text{-}ClC_6H_4).CCl_2.CHCl_2$ to *C. granaria*.

The chlorophenyl-ethane derivatives were assayed, using deposits from acetone solutions, and the results are shown in Table 2. Unfortunately, the whole series could not be tested systematically and in several cases the potency relative to the most toxic compound, $(4\text{-}ClC_6H_4).CCl_2.CHCl_2$, has been calculated indirectly. Since there are appreciable differences of slope between the various regression lines comparative toxicities cannot be expressed as true relative potencies, but the results are such that a comparison of the ED 50's will give an approximate idea of the general order of insecticidal activity.

The relative magnitudes of the ED 50's were as follows:

(4-Cl.C_6H_4) CCl_2.CCl_3	0·03	(4-Cl.C_6H_4) CHCl.CCl_3	0·47
(4-Cl.C_6H_4) CCl_2.$CHCl_2$	1·00	(4-Cl.C_6H_4) CHCl.$CHCl_2$	0·33
(4-Cl.C_6H_4) CCl_2.CH_2Cl	0·15	(4-Cl.C_6H_4) CHCl.CH_2Cl	0·07
		(4-Cl.C_6H_4) CHCl.CH_3	0·02

(4-Cl.C_6H_4) CCl:CCl_2	0·34
(4-Cl.C_6H_4) CCl:CHCl	0·11
(4-Cl.C_6H_4) CCl:CH_2	0·04
(4-Cl.C_6H_4) CH:CH_2	0·04

From an examination of the list of relative potencies of the (4-chlorophenyl)-ethane series it seems that the toxic constituent of 'Lucex' is probably the —CCl_2CHCl_2 compound, although compounds like the —$CHCl.CCl_3$, —$CHCl.CHCl_2$, and —$CCl:CCl_2$ may be present in sufficient quantities to exert insecticidal action.

Discussion

As pointed out by Martin & Wain (1944*a*) the requirements of an insecticide involve: (1) ability to penetrate and to concentrate at the site of action, (2) adequate stability to reach the site of action, and (3) the toxic mechanism at the site of action. The inference of laws relating toxic action and chemical structure would of necessity imply the elucidation of the third requirement. A chemical compound is toxic if it causes disturbances, often lethal, in certain physiological systems and it is only effective when at the site of action in sufficient concentration. The usual bio-assay systems measure the 'insecticidal effectiveness' of compounds by correlating the mortality observed with the concentration of the substance presented to the insect, i.e. the concentration external to the insect. The concentration at the site of action is not known, and unless it can be safely inferred that the same proportionality exists between amounts presented and amounts at the site of action for two compounds, then even the relative concentrations interfering with physiological systems are not known and it is thus difficult to see how the toxic action of the two compounds can be strictly compared. Thus, in a bio-assay system, the factors concerning ability to penetrate and to reach the site of action may be of overriding importance, and in formulating relationships between structure and activity it must be remembered that a measured biological response is a summation of the three requirements mentioned. Since the concentrations at the site of action cannot be measured, and since no definite relationship between internal and external concentration has been ascertained, only very general conclusions can be made with regard to structure and activity, it being remembered that probably in most cases divergent results are due to the biological measurements being non-comparable.

The mode of action of D.D.T. has not yet been elucidated, although Bodenstein

(1946) has demonstrated that D.D.T. causes muscular convulsions by affecting the peripheral nervous system. Roeder & Weiant (1946) have concluded that D.D.T. poisoning, in the cockroach, is due to an intense and patternless bombardment of motor neurones by trains of impulses originating in sensory endings; their experiments also suggest that D.D.T. does not have this effect on all sensory structures, but acts specifically on some particular group of sense organs which have not yet been identified. It thus appears that D.D.T. induces a state of hyperactivity in the insect nervous system which results in death by exhaustion; such a toxic mechanism would account for the slow action of D.D.T. and the unusually flat probit-dosage lines obtained during assay. Although the site of action has now been more or less demonstrated, the problem considered by Martin & Wain (1944*b*), Laüger *et al.* (1944), and Busvine (1945) is still essentially the same.

The results of the investigations recorded in this paper indicate that the insecticidal effectiveness of D.D.T. is due to the combination of the two chlorophenyl groups and the trichloroethane group. Modifications of the latter group result in loss of toxicity and the compounds possessing $=CH.CCl_3$, $=CH.CHCl_2$ and $=CH.CH_2Cl$ indicate a relationship between effectiveness and degree of chlorination. The (4-chlorophenyl)-ethane series, containing only one (4-chlorophenyl) group, are considerably less toxic than D.D.T. to *Calandra*, and the general trend of toxicity in this series shows a correlation with the degree of chlorination on the ethane part of the molecule, although complete chlorination results in a virtually non-toxic compound. This latter compound is incapable of losing hydrogen chloride. Following from the Laüger *et al.* hypothesis the reduction of toxicity may be attributed to the loss of one 4-chlorophenyl group, whereas the Martin & Wain hypothesis suggests that the loss of a 4-chlorophenyl group may result in reduction of lipoid solubility, and the relation between toxicity and degree of chlorination may be associated with the ease of hydrogen chloride liberation from the ethane group. There is also a direct relationship between chlorine content and toxicity in the corresponding ethylenic groups. Dehydrochlorination of $(4Cl.C_6H_4).CCl=CCl_2$ is not possible as in the other compounds and the insecticidal activity may be due to the liberation of hydrogen chloride in some other way or to the ethylenic linkage. The comparatively non-toxic ethylenic derivatives of D.D.T., when contrasted with the compound $(4Cl.C_6H_4)\,CCl=CCl_2$, suggests that spatial configuration of the molecule may also be of importance.

Conclusions

On a weight basis (mg./ml.), D.D.T. is more toxic than its *p*-bromo-derivative which is more toxic than its *p*-iodo-derivative, and when concentrations are measured on the molar scale D.D.T. and its *p*-bromo-analogue are approximately equally effective, whereas the *p*-iodo-analogue has about half their toxicity.

D.D.T. analogues, in which the $=CH.CCl_3$ group is modified, show a loss of

effectiveness, only D.D.D. having moderate toxicity. The ethylenic series are comparatively non-toxic.

The chlorinated (4-chlorophenyl)-ethanes are considerably less toxic than D.D.T., but show some correlation between chlorine content and insecticidal activity.

This work has been carried out under a special Research Grant from the Agricultural Research Council to whom grateful acknowledgement is made. The author is also indebted to Dr H. Martin for his interest and advice in carrying out this work, and also to Dr D. Woodcock and Dr R. L. Wain for the preparation of the compounds used.

REFERENCES

BODENSTEIN, D. (1946). Investigation on the locus of action of D.D.T. in flies (*Drosophila*). *Biol. Bull. Woods Hole*, **90**, 148.

BUSVINE, J. R. (1945). Insecticidal action of D.D.T. *Nature, Lond.*, **156**, 169.

FINNEY, D. J. (1947). *Probit Analysis*. Cambridge University Press.

LAÜGER, P., MARTIN, H. & MÜLLER, P. (1944). Über Konstitution und toxische Wirking von natürlichen und neuen synthetischen insektentötenden Stoffen. *Helv. Chim. Acta*, **27**, 892.

MARTIN, H. & SHAW, H. (1946). Developments in methods and materials for the control of plant pests and diseases in Germany. *B.I.O.S. Final Report*, no. 1095. London: H.M. Stationery Office.

MARTIN, H., STRINGER, A. & WAIN, R. L. (1943). The qualitative examination of insecticidal properties. Progress report—1943. *Rep. agric. hort. Res. Sta., Bristol*, p. 62.

MARTIN, H. & WAIN, R. L. (1944*a*). The qualitative examination of insecticidal properties. Progress report—1944. *Rep. agric. hort. Res. Sta., Bristol*, p. 121.

MARTIN, H. & WAIN, R. L. (1944*b*). Insecticidal action of D.D.T. *Nature, Lond.*, **154**, 512.

ROEDER, K. D. & WEIANT, E. A. (1946). Site of action of D.D.T. in the cockroach. *Science*, **103**, 304.

STRINGER, A. (1948). A simple method of assaying contact toxicities of insecticides, with results of tests of some organic compounds against *Calandra granaria* L. *Ann. appl. Biol.* **36**, 218.

WOODCOCK, D. (1949). Insecticidal activity and chemical constitution. Part I: Chlorinated *p*-chloroethylbenzenes. *J. chem. Soc.* **48**, 203.

(*Received* 9 *September* 1948)

A SIMPLE METHOD FOR ASSAYING CONTACT TOXICITIES OF INSECTICIDES, WITH RESULTS OF TESTS OF SOME ORGANIC COMPOUNDS AGAINST *CALANDRA GRANARIA* L.

By A. STRINGER

Long Ashton Research Station, Bristol

(With 6 Text-figures)

A simple method of testing the contact action of insecticides either as residual dry deposits or as oil films is described. Tests with crystalline deposits of D.D.T. indicated that this method of assay was not sufficiently sensitive for practical use, but tests with oil films could be used. Some of the factors governing the toxicity of D.D.T. in the two systems are discussed, and the results of tests with several organic compounds are given.

Introduction

Attempts were made to develop simple methods of assaying insecticides which would give consistent and reliable estimates of their relative toxicities. It was desirable that these laboratory tests should resemble practice in that the insect was in contact with either a residual dry insecticide deposit or an oil film. The simplest technique for testing contact toxicity involves the presentation of the insecticide to the insect in a standard reproducible way, and the deposition of a known weight of insecticide in a volatile solvent on to a standard substrate is obviously the simplest method. Barnes (1945) impregnated filter-papers with D.D.T. by pipetting on to them a known volume of acetone solution. Proverbs & Morrison (1947) used filter-papers which had been immersed in acetone solutions and also glass vials coated with insecticide; the vials were filled with acetone solution, emptied, inverted and left to dry. Oil-film techniques have been described by Tattersfield & Potter (1943) and by Parkin & Green (1943) for the evaluation of pyrethrum preparations. In each case a thin hard filter-paper (Whatman No. 544) was considered the best substrate and the required dosage was obtained by spraying the paper in a specially constructed spray tower (Potter, 1941).

The object of the investigations described in this paper was to examine the dry deposit and oil-film methods when the dosages were applied without the use of special apparatus.

Description of technique

Test insects. The insects used were adult grain weevils (*Calandra granaria* L.) reared on wheat in glass jars at 25° C.; main cultures started with 200 adults 0–1 week old and these weevils were removed after 4 weeks; the adults which

emerged subsequently were removed at weekly intervals and transferred in batches of 200 to fresh wheat. The insects were used for test purposes when 2–4 weeks old and were starved overnight before use.

Preparations of filter-papers. It was decided to use No. 1 Whatman filter-papers, 7 cm. in diameter, and it was found that 0·5 ml. of acetone just flooded the paper when the pipette was moved in a spiral manner above the paper. Thus, using insecticide-acetone solutions of known concentration deposits of known weight were obtained after the solvent evaporated.

In the oil-film technique the insecticide was dissolved in oil, generally P31 petroleum oil (specification; Robinson, 1942), and a known volume was deposited at the centre of the paper from a micro-pipette. The oil was allowed to diffuse outwards, and although a period of 6 hr. appeared to be sufficient for the oil to spread over the paper, the oil film was allowed to age 24 hr. before it was used for test purposes.

Results of tests with dry deposits of insecticide

D.N.C. Tests with D.N.C. (di-nitro-*ortho*-cresol) were satisfactory. Seven dosage levels were used (Table 4), varying from 3·8 to 15·2 moles $\times 10^{-7}$ per filter-paper, and the calculated probit-log (dosage $\times 10^{7}$) regression line was

$$y = 4{\cdot}862x + 0{\cdot}650,$$

with $\chi^2 = 3{\cdot}49$ for 4 degrees of freedom.

D.D.T. Tests with D.D.T. gave unexpected results; inspection of the percentage kills (Table 1) indicates that there was no difference of toxic action over the range of dosages used (0·0098 – 0·315 mg./cm.^{2}), although the highest is 32 times greater than the lowest dosage.

Table 1. *Tests with D.D.T. against* Calandra granaria

	D.D.T. (mg./cm.^{2})	No. of *Calandra*	Percentage killed at 24 hr.	48 hr.	72 hr.	96 hr.	120 hr.	144 hr.
I	0·315	26	4	12	19	39	81	89
	0·158	25	0	8	24	44	56	64
	0·0788	23	4	9	26	48	70	74
	0·0394	25	0	4	28	52	68	80
	0·0197	25	0	12	20	40	68	88
	0·0098	25	0	4	16	48	68	84
II	0·315	24	0	4	20	42	58	79
	0·158	24	0	0	8	29	63	83
	0·0788	27	0	7	11	41	56	78
	0·0394	25	0	8	16	32	60	76
	0·0197	25	0	12	32	60	68	84
	0·0098	25	0	12	28	52	80	92
Control	—	50	0	0	0	0	2	8

In further tests the dosage range was extended so that the highest ($3{\cdot}15 \times 10^{-1}$ mg./cm.^{2}) was 100,000 times as great as the lowest dosage ($3{\cdot}15 \times 10^{-6}$ mg. cm.^{2}).

Figs. 1–3 show the relation between probit mortality and log-weight deposit. The three separate tests demonstrate that the bending of the probit-log weight regression line at approximately 3×10^{-4} mg./cm.^{2} is real and consistent. The slope of the regression line obtained for dosages below 3×10^{-4} mg./cm.^{2} is very low, approximately 0·5–1·0, and indicates that this method of assay is not sufficiently sensitive for practical use; also, such low dosages would necessitate the use of solutions of concentration less than 0·01 % (w/v).

Whatman filter-papers No. 1, moderately coarse and thick, and No 50, hard and thin, were impregnated with D.D.T. from acetone and alcohol solutions, five dosage levels being used. The results are recorded in Table 2, and the percentage

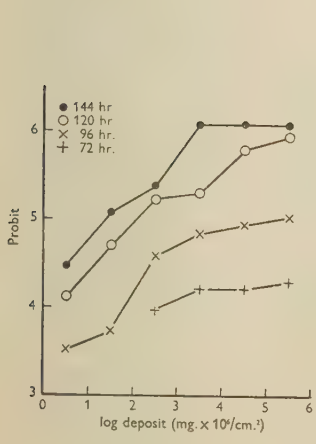


Fig. 1

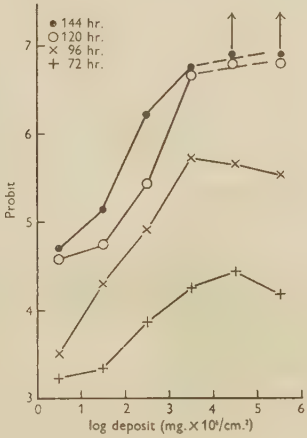


Fig. 2

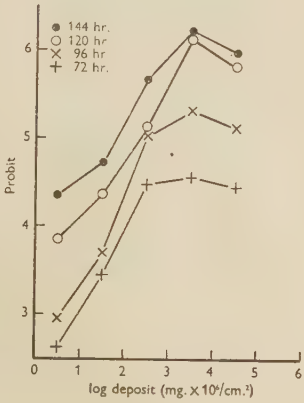


Fig. 3

Figs. 1, 2 and 3. The toxicity to *C. granaria* of crystalline D.D.T. deposits on No. 1 Whatman filter-paper.

kills transformed into angles are analysed in Table 3. The factors for time, concentration, treatments, and the interaction for treatments-concentration are significantly different. Inspection of Table 2 indicates that the action of D.D.T. is similar at dosages of $1{\cdot}3 \times 10^{-2}$ mg./cm.^{2}, and higher, for both types of solvent and substrates. Below this dosage, treatment differences appear, especially on the No. 1 Whatman papers impregnated with D.D.T. from alcohol. Deposits from acetone on both No. 1 and No. 50 papers are not substantially different, and deposits from alcohol show pronounced differences at the lower dosages, the kills being higher on the harder and thinner paper. The No. 50 paper shows little or no difference of kill between deposits from alcohol and acetone, whereas the coarser and thicker No. 1 paper gives higher kills at lower dosages with deposits from acetone than from alcohol.

Proverbs & Morrison (1947) have recorded the bending of the probit-dosage regression line when using the impregnated filter-paper method against *Drosophila*

TABLE 2. *Toxicity of D.D.T. deposits from acetone and alcohol solutions against* Calandra granaria

Treatment	Dosage	*n*	Percentage killed at 48 hr.	72 hr.	96 hr.	120 hr.
Deposit from acetone	*a*	50	12	50	78	83
solution on No. 1	*b*	51	20	57	78	94
Whatman	*c*	50	14	54	84	88
	d	50	6	58	74	84
	e	50	2	12	36	56
Acetone solution on	*a*	50	12	46	70	80
No. 50 Whatman	*b*	49	12	45	76	88
	c	51	6	39	67	88
	d	49	16	49	65	92
	e	50	4	22	62	78
Alcohol solution on	*a*	40	5	50	83	85
No. 1 Whatman	*b*	56	18	66	77	89
	c	50	10	28	56	70
	d	49	2	25	43	57
	e	50	0	0	4	18
Alcohol solution on	*a*	50	18	64	86	94
No. 50 Whatman	*b*	50	22	60	90	94
	c	48	17	58	77	90
	d	51	20	57	77	86
	e	51	6	24	61	75
Control	—	200	0	1	3	3

Dosage levels: $a = 1{\cdot}30 \times 10^{-1}$, $b = 4{\cdot}33 \times 10^{-2}$, $c = 1{\cdot}30 \times 10^{-2}$, $d = 4{\cdot}33 \times 10^{-3}$, $e = 1{\cdot}30 \times 10^{-3}$ mg.cm.$^{-2}$

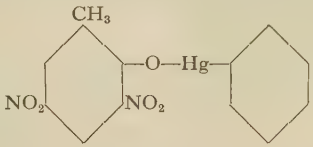
TABLE 3. *Analysis of results of action of D.D.T. deposits, from acetone and alcohol, on* Calandra granaria

Items	D.F.	Mean square	Probability
Time	3	8115	$< 0{\cdot}001$
Concentration	4	1384	$< 0{\cdot}001$
Treatments	3	742	$< 0{\cdot}001$
Time-concentration	12	34	0·05–0·01
Time-treatments	9	21·7	$< 0{\cdot}05$
Concentration-treatments	12	150·1	$< 0{\cdot}001$
Error	36	15·0	—
Total	79	—	—

melanogaster, and they suggested that solutions of different concentrations deposit crystals of different sizes, thus affecting the mortality. Barnes (1945) found differences of toxicity with D.D.T. crystallized from acetone and from odourless

distillate, when tested against bed-bugs, and suggested that the initial toxicity of the surface is determined to some extent by the volatility of the solvent used. The experiments with deposits from alcohol and acetone on No. 1 and No. 50 Whatman papers against *Calandra* also indicate that the solvent and the nature of the substrate exert an effect upon the deposit which is reflected in the degree of mortality. It is obvious that the availability of the deposit depends upon the crystal size and shape, the distribution over the filter-paper, and also upon the insect-insecticide relationship. Since deposits of the order 3×10^{-6} mg./cm.^{2} produce appreciable kills, it is clear that only extremely small dosages of D.D.T. are required to kill each *Calandra* adult, for example, a total deposit of 0·11 mg. from acetone on a No. 50 paper gave nearly 100% kill, and even if the weevils picked up all the D.D.T. present the average dosage would be 0·002 mg. per weevil. The data also demonstrate that the rate of toxic action on the insect is slow, so that at high dosages the quantity of D.D.T. available is in excess of the amount required to kill and the insect picks up the D.D.T. at a rate independent of the weight present, so that at these dosages the kill is not increased by additional increments of insecticide. At lower dosages where there is a regression of kill on weight deposit the sampling rate is below the maximum level, and it is here that differences in crystal size and distribution affect the mortality.

Phenyl mercury derivative of D.N.C. The sample used was supplied by Messrs Lunevale Products Ltd. and had the molecular structure



The probit lines were parallel when assayed against D.N.C. (Table 4):

$$y = 4{\cdot}754x + 0{\cdot}579 \text{ (phenyl mercury D.N.C.)},$$

$$y = 4{\cdot}754x + 0{\cdot}752 \text{ (D.N.C.)};$$

where $x = \log (\text{moles} \times 10^{-7})$. Analysis of χ^2 gave $\chi^2 = 0{\cdot}65$ for 1 D.F. for parallelism of regression lines and $\chi^2 = 0{\cdot}763$, 9 D.F., for the residual heterogeneity.

$$\text{ED } 50 \text{ (phenyl mercury D.N.C.)} = 8{\cdot}51 \times 10^{-7} \text{ moles/paper},$$

and

$$\text{ED } 50 \text{ (D.N.C.)} = 7{\cdot}83 \times 10^{-7} \text{ moles/paper}.$$

At the 5% probability level $M = 0{\cdot}0464 \pm 0{\cdot}0625$ ($g = 0{\cdot}0381$) and the relative potency of D.N.C. with respect to the phenyl mercury derivative is 1·11 with fiducial limits of 1·28 and 0·96. The toxicities of the two compounds are not significantly different; the inclusion of the phenyl mercury group into the D.N.C. molecule apparently did not alter its toxicity and on a weight basis (mg./paper) D.N.C. is 2·6 times as toxic.

Phenyl diazopiperidine, tetranitrocarbazole, and 4-chlorophenyl chloromethyl sulphone. These compounds were used as insecticides in Germany during the war (Martin & Shaw, 1946). Tetranitrocarbazole, a compound with chemical structure similar to phenothiazine, was developed for application to vines. It was used as a dust or dispersible powder and appeared to exercise a selective action against *Clysia ambiquella* and *Polychrosis botrana*. Samples of this compound supplied by Dickinson & Son, crude and pure 1:3:6:8-tetranitrocarbazole, were non-toxic when tested against *Calandra granaria*.

TABLE 4. *Toxicity of phenyl mercury D.N.C. and D.N.C*

Phenyl mercury D.N.C.				D.N.C.			
Moles × 10^{-7} per paper	No. of *Calandra*	No. killed	Percentage killed	Moles × 10^{-7} per paper	No. of *Calandra*	No. killed	Percentage killed
4·74	24	4	17	3·79	25	3	12
6·32	25	5	20	5·69	26	5	19
7·90	25	9	36	7·58	25	13	52
9·48	25	13	52	9·48	24	14	58
11·06	25	15	60	11·38	25	20	80
12·64	25	20	80	13·27	26	23	89
14·22	25	21	84	15·16	27	26	96
15·80	24	22	92				

Exposure time, 24 hr.

TABLE 5. *Toxicity of phenyl diazopiperidine and D.N.C.*

Phenyl diazopiperidine				D.N.C.			
Moles × 10^{-7} per paper	No. of *Calandra*	No. killed	Percentage killed	Moles × 10^{-7} per paper	No. of *Calandra*	No. killed	Percentage killed
10·6	60	0	0	5·1	59	2	3
21·1	59	1	2	10·1	55	17	31
31·7	59	5	8	15·2	60	43	72
42·3	60	11	18	20·2	59	46	78
52·9	60	21	35	25·3	58	56	97
63·4	60	21	35	30·3	60	58	97
74·0	60	28	47	35·4	61	60	98
84·6	60	30	50	40·4	60	60	100
95·1	61	35	58				
105·7	59	41	70				

4-chlorophenyl chloromethyl sulphone (Lauseto-neu) was used for the control of body-lice, and Busvine (1946) reports that it was more toxic to both lice and bed-bugs than D.D.T. This compound had no insecticidal action against *Calandra*.

Phenyl diazopiperidine (Dizan) has been used against the forestry pests *Lymantria monacha* and *Bupalus piniarus* and also as a cockroach poison. This compound was tested against D.N.C. and the results are shown in Table 5. The probit regression lines are not parallel, being

$$y = 3{\cdot}405x - 1{\cdot}457 \text{ (phenyl diazopiperidine)},$$

$$y = 4{\cdot}917x - 0{\cdot}377 \text{ (D.N.C.)}.$$

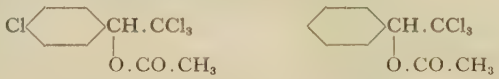
Since the probit lines are not parallel the toxicities cannot be expressed as a relative potency ratio, but consideration of the ED 50's and 95's show that D.N.C. is considerably more toxic than phenyl diazopiperidine.

	Phenyl diazopiperidine	D.N.C.
ED 50	7·87 moles/paper	1·24 moles/paper
5 % level fiducial limits	5·98–8·22	1·19–1·30
ED 95	23·93 moles/paper	2·68 moles/paper
5 % level fiducial limits	18·37–31·19	2·52–2·85

Hexamethylcyclohexane and hexaethylcyclohexane. Slade (1945) has suggested that the physiological activity of γ-hexachloro*cyclo*hexane is due to its antagonism towards the metabolite *i*-inositol, which is considered to have the same spatial configuration. The similar compounds hexamethyl- and hexaethyl*cyclo*hexane were found to possess negligible toxic action against *Calandra granaria*.

Tetranitrodiphenol, Di-2-pyridylamine, Acenaphthylene dibromide, and Acenaphthylene dithiocyanate. These compounds were supplied by the Chemical Research Laboratory, Teddington, and proved to be non-toxic against *Calandra*. Tetranitrodiphenol was tested because its chemical structure resembled D.N.C., and di-2-pyridylamine the carbazole compound. Acenaphthylene dibromide was used because of its ability to liberate hydrogen bromine and acenaphthylene thiocyanate because of the known insecticidal action of thiocyanate groups.

Acetyl derivatives of phenyl trichloromethyl carbinol.



These two compounds, which had shown toxic properties in preliminary experiments against red spider, were non-toxic to *Calandra*.

Results of tests with insecticidal oil films

D.D.T.-oil. It was found with oil films of P 31 petroleum oil that dosages below 0·06 ml. per filter-paper (0·00155 ml./cm.^{2}) were insufficient for a good spread, and that dosages above 0·16 ml. per filter-paper (0·00416 ml./cm.^{2}) had pronounced toxic action (Fig. 4). Hewlett (1947) has shown that the symptoms observed in weevils poisoned by P 31 oil are similar to those produced by asphyxiation and infers that the P 31 oil probably exerts only a mechanical effect, causing suffocation by blocking the spiracles and tracheae. Fig. 5 shows the probit mortality against log deposit for 0·5 % (w/v) D.D.T. and it is seen that the curves are considerably concave and cannot be represented linearly. A combination of two linear regression lines can be made to fit the data, suggesting that the curve represents the sum effect of the two independent regression lines of kill by D.D.T.-oil solution and the kill due to the mechanical effect of P 31 oil. The position of the probit lines for the P 31 oil alone when compared with their corresponding D.D.T.-oil curves does not support the idea of two independent intersecting probit lines (Finney, 1947). The form of the

curves suggests that the slope steadily increases even when the dosage is low and P31 oil alone has little or no toxic effect. Stringer (1948) has noted that the slope of the dosage regression line is dependent upon the bioassay system used; in this

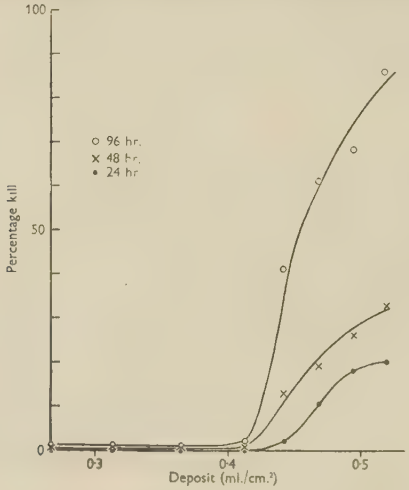


Fig. 4. Toxicity to *C. granaria* of P31 oil films.

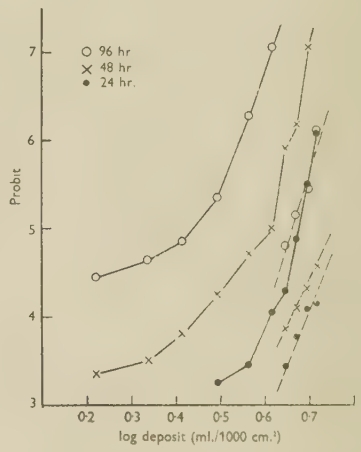


Fig. 5. Toxicity to *C. granaria* of 0·5% D.D.T. in P31 oil. (Broken lines show toxicity of P31 oil films.)

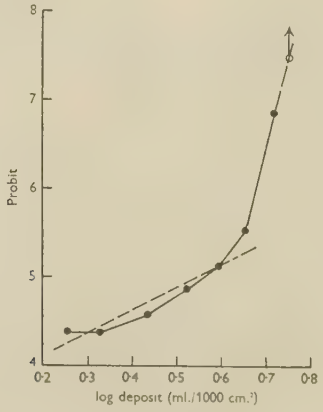


Fig. 6. Toxicity to *C. granaria* of 1% D.D.T. in P31 oil.

case appreciable differences of film thickness could be considered as altering the system, and if increased film thickness resulted in increased availability of the D.D.T. in solution then an increase of slope would be expected and the result over a range of dosages would be a curve of the type obtained.

Fig. 6 shows similar results with 1% D.D.T. in P31 oil, dosages 0·0018–0·0060 ml./cm.^{2} The calculated regression line fitting the lower dosages, 0·0018–0·0045 ml./cm.^{2}, is $y = 579x + 3·598$ for $\chi^2 = 1·639$, 4 D.F., and $x = \log$ (ml./1000 cm.^{2}).

To eliminate the differential effect of oil-film thickness it was decided to use one dosage and vary the concentration of the insecticide. The standard dosage used was nominally 0·12 ml. per No. 1 7 cm. Whatman filter-paper, and it was found that the mean deposit was 0·1006 ± 0·0010 g. per paper or 0·118 ± 0·001 ml. per paper (P31 sp.gr. = 0·853 at 20° C.). Tests with both D.D.T. and D.N.C. gave adequate regression lines (Tables 6 and 7).

TABLE 6. *Toxicity to* Calandra granaria *of D.N.C. in P31*

Concentration (mg./ml.)	No. of *Calandra*	No. killed	Percentage killed
5	49	47	96
4	50	46	92
3	50	45	90
2	49	38	77
1	49	23	47
0·5	50	12	24
0	150	3	2

Dosage: 0·12 ml./paper. Exposure time, 48 hr. Regression equation $y = 2·551x + 2·407$, where $x = \log$ (mg. × 10/ml.). $b = 2·551 \pm 0·277$. $\chi^2 = 0·414$ for $n = 2$. $m = 1·016 \pm 0·041$. E.D. 50 = 1·04 mg./ml. and fiducial limits at 5% level are 1·25 and 0·86 mg./ml.

TABLE 7. *Toxicity to* Calandra granaria *of D.D.T. in P31*

Concentration (mg./ml.)	No. of *Calandra*	No. killed	Percentage killed
5·0	62	50	81
4·5	60	42	70
4·0	60	37	62
3·5	58	31	53
3·0	59	38	64
2·5	29	10	34
2·25	62	31	50
2·0	60	21	35
1·75	60	27	45
1·50	30	6	20
0	190	5	2·6

Dosage, 0·12 ml./paper. Exposure time, 96 hr. $y = 2·333x + 1·678$, where $x = \log$ (mg. × 10/ml.). $b = 2·333 \pm 0·352$. $m = 1·424 \pm 0·059$. $\chi^2 = 7·228$ for $n = 7$. ED 50 = 2·66 mg./ml. and fiducial limits at 5% level are 3·43 and 2·06 mg./ml.

From the results of tests carried out on many compounds it is concluded that either one or both of the methods described can be used to estimate the relative toxicities of contact insecticides. In the case of compounds tested against D.D.T. the oil-film method must be used.

Velsicol 1068 (*Chlordane*). The sample used was a 90% concentrate, supplied by the Velsicol Corporation, and was assayed against D.D.T. by the oil-film method.

The results are shown in Table 8. The probit lines were not parallel (χ^2 for 1 D.F. $=4{\cdot}738$) and the equations for the two compounds were:

$$y = 1{\cdot}387x + 3{\cdot}319 \text{ (Velsicol 1068)},$$
$$y = 2{\cdot}150x + 3{\cdot}042 \text{ (D.D.T.)};$$

$x = \log (10 \times \text{mg./ml.})$, and the corresponding χ^2 values were 2·488 for 8 D.F. and 2·538 for 5 D.F.

Over the range of concentrations used D.D.T. was considerably more toxic than Velsicol 1068, the ED's 95, 50 and 25 for D.D.T. being 4·74, 0·81 and 0·40 mg./ml. and for Velsicol 1068, 25·00, 1·63, and 0·53 mg./ml. Thus the relative potencies of Velsicol 1068 with respect to D.D.T. at 95, 50 and 25% kill are 0·19, 0·50 and 0·74.

TABLE 8. *Toxicity of Velsicol* 1068 *and D.D.T. in P*31 *oil*

Velsicol 1068				D.D.T.			
Concentration (mg./ml.)	No. of *Calandra*	No. killed	Percentage killed	Concentration (mg./ml.)	No. of *Calandra*	No. killed	Percentage killed
0·45	50	16	32	0·5	50	20	40
0·45	50	15	30	0·75	50	27	54
0·90	51	21	41	1·0	50	28	56
0·90	51	21	41	1·5	55	45	82
1·8	50	29	58	2·0	50	42	84
1·8	54	33	61	3·0	48	43	90
3·6	56	42	75	4·0	50	46	92
3·6	49	35	71				
5·4	51	37	73				
5·4	50	41	82				

Control mortality, 11·5%. Dosage, 0·12 ml./paper.

TABLE 9. *Toxicity of Toxaphene and D.D.T. in P*31 *oil*

Toxaphene				D.D.T.			
Concentration (mg./ml.)	No. of *Calandra*	No. killed	Percentage killed	Concentration (mg./ml.)	No. of *Calandra*	No. killed	Percentage killed
20	50	45	90	4	50	42	84
10	48	36	75	3	48	40	87
8	53	29	55	2·5	50	40	80
4	52	17	33	2	50	34	68
2	50	11	22	1·5	50	33	66
1	50	5	10	1	50	30	60
0·5	51	2	4	0·75	51	23	45
				0·5	52	18	35

Control mortality, 8%. Dosage, 0·12 ml./paper. Exposure time, 120 hr.

Toxaphene is a chlorinated camphene, and the sample used was supplied by Hercules Powder Co. Solutions in P31 oil were assayed against D.D.T. by the oil-film method (Table 9). The probit mortality data are:

(1) Toxaphene $y = 2{\cdot}030x + 1{\cdot}325$,
$m = 1{\cdot}810$;

(2) D.D.T. $y = 2{\cdot}030x + 2{\cdot}940$,
$m = 1{\cdot}015$.

The probit lines were parallel, χ^2 for 1 D.F. being 2·54, and $M_{21} = 0{\cdot}7955 \pm 0{\cdot}0594$. Thus the relative potency of Toxaphene with respect to D.D.T. was 0·16 with fiducial limits at the 5% probability level of 0·12–0·21.

Diethyl p-*nitro-phenyl thiophosphate* (E605). The sample used was a crude ester from Schrader's laboratory labelled E605 Rohprodukt (Martin & Shaw, 1946). The compound appeared to be immiscible with P31 oil and was assayed against D.D.T. in castor oil. Table 10 gives the results of the test, using 0·25 g. of castor-oil solution per filter-paper.

TABLE 10. *Toxicity of* E605 *and D.D.T. to* Calandra granaria

Treatment	Concentration (mg./ml.)	No. of *Calandra*	Percentage killed at 20 hr.	48 hr.	70 hr.	96 hr.	120 hr.
E 605 in	2·5	101	5	39	63	87	100
castor	5·0	100	9	45	73	89	100
oil	10·0	100	19	53	78	94	100
	20·0	111	31	65	86	96	100
D.D.T.	2·5	102	0	6	15	30	53
in castor	5·0	101	0	10	26	43	58
oil	10·0	102	0	28	44	57	75
	20·0	100	0	28	50	68	83
Castor oil	—	200	0	0	1	4	12

The probit regression lines obtained from the kills after a 70 hr. exposure period were:

$$y = 0{\cdot}800x_1 + 5{\cdot}018 \text{ (E605)},$$

$$y = 1{\cdot}238x_2 + 3{\cdot}475 \text{ (D.D.T.)};$$

x = log (mg./ml.), and the corresponding χ^2 for 2 D.F. were 0·217 and 1·733.

Assuming that the lines were parallel, the modified equations were:

$$y = 1{\cdot}024x_1 + 4{\cdot}835, \quad m_1 = 0{\cdot}2588 \text{ (E605)},$$

$$y = 1{\cdot}024x_1 + 3{\cdot}664, \quad m_2 = 1{\cdot}3052 \text{ (D.D.T.)},$$

and the analysis of χ^2 gave

	D.F.	S.S.	Mean square
Parallelism of regressions	1	2·368	2·368
Residual heterogeneity	4	1·950	0·488
Total	5	4·318	—

$M_{12} = 1{\cdot}0464 \pm 0{\cdot}1660$, and thus the relative potency of E605 with respect to D.D.T. was 11·1 with fiducial limits at the 5% probability level of 6·0–29·7.

This work has been carried out under a special Research Grant from the Agricultural Research Council to whom grateful acknowledgement is made.

REFERENCES

BARNES, S. (1945). The residual toxicity of D.D.T. to bed-bugs. *Bull. ent. Res.* **36**, 273.

BUSVINE, J. R. (1946). New synthetic contact insecticides. *Nature, Lond.*, **158**, 22.

FINNEY, D. J. (1947). *Probit Analysis*, p. 142. Cambridge University Press.

HEWLETT, P. S. (1947). The toxicities of three petroleum oils to the grain weevils. *Ann. appl. Biol.* **34**, 575.

MARTIN, H. & SHAW, H. (1946). Developments in methods and materials for the control of plant pests and diseases in Germany. *B.I.O.S. Final Report*, No. 1095. London: H.M. Stationery Office.

PARKIN, E. A. & GREEN, A. A. (1943). A film technique for the biological evaluation of pyrethrum-in-oil insecticides for use against stored product insects in warehouses. *Ann. appl. Biol.* **30**, 279.

POTTER, C. (1941). A laboratory spraying apparatus and technique for investigating the action of contact insecticides. *Ann. appl. Biol.* **28**, 142.

PROVERBS, M. D. & MORRISON, F. O. (1947). The relative toxicity of D.D.T. and related organic molecules. *Canad. J. Res.* **25**, D 12.

ROBINSON, G. G. (1942). The penetration of pyrethrum through the cuticle of the tick, *Ornithodorus moubata* Murray. *Parasitology*, **34**, 113.

SLADE, R. E. (1945). Hurter Memorial Lecture. *Chem. & Ind.* **64**, 314.

STRINGER, A. (1948). Relation between bioassay systems and the values found for toxicity of D.D.T. *Ann. appl. Biol.* **35**, 527.

TATTERSFIELD, F. & POTTER, C. (1943). Biological methods of determining the insecticidal values of pyrethrum preparations (particularly extracts in heavy oil). *Ann. appl. Biol.* **30**, 259.

(*Received* 9 *September* 1948)

BIOASSAY SYSTEMS FOR THE PYRETHRINS

I. WATER-BASE SPRAYS AGAINST *AËDES AEGYPTI* L. AND OTHER FLYING INSECTS

By A. B. P. PAGE, A. STRINGER* and R. E. BLACKITH

Imperial College of Science and Technology Field Station, Silwood Park, Sunninghill, Berks

(With 16 Text-figures)

A bioassay system for the pyrethrins is discussed in which the quantity of toxicant that flying insects accumulate is measured, as well as the responses of the insects. The determination of spray pick-up from water-base sprays is described, and the use of primary responses, such as activation and paralysis, in biological assays is advocated. The test system is discussed in detail for *Aëdes aegypti* L. and the special characteristics of some other species of flying insects noted. It is considered that the location of the site of action of the pyrethrins under these conditions is at the peripheral nervous system of the test insects.

Water-base sprays and *Aëdes aegypti* L.

Relation between toxicity and pick-up of insecticides applied as aqueous sprays

Much published work has been based on the tacit assumption that the quantity of insecticide accumulated by an insect flying in a spray is proportional to the concentration of insecticide in the spray fluid and to the volume sprayed into the air space occupied by the insects. Published statements that wasps, horseflies and blowflies are more sensitive than houseflies to pyrethrins, based on the concentrations used, ignore the fact that the first-mentioned insects, which are hairy, pick up far more spray liquid in flight or when stationary than the houseflies. We have shown that the weight of insecticide acting on the insect is proportional to the product of the volume of spray liquid accumulated by the insect and the concentration of insecticide in the liquid.

If the volume of liquid picked up by the insect is plotted against the concentration of insecticide in the spray liquid, the points representing the acquisition of a constant quantity of insecticide by the insect should then lie on a rectangular hyperbola. This has been shown to be true for a number of insects, using their responses to the weights of pyrethrins accumulated. Any variation of the conditions which alters the quantity of spray liquid accumulated, such as a change of nominal spray dosage, or humidity, also alters the concentration at which any particular response occurs and alters it in such a way that the threshold weight of the insecticide causing the response is maintained.

* Now at Department of Agriculture and Horticulture, Long Ashton Research Station, Bristol University.

The bulk of the work on flying insects has been done with *A. aegypti* L. as a test insect, and with the pyrethrins as the activating and paralysing agent in a water-base emulsion. The emulsion was prepared from an extract containing 20% pyrethrins kindly supplied by Dr T. F. West of Messrs Stafford Allen and Sons, by dissolving the extract in ethylene glycol monoethyl ether ('Cellosolve') so as to form a 1% solution and incidentally to remove the bulk of the associated waxy material. This stock 1% solution readily emulsifies in any desired proportion with water.

The method of determination of the amount of spray accumulated by the insects depends on the inclusion in the spray of aluminium chloride.

Determination of aluminium in spray deposits on flying insects

Spray procedure. The insects to be sprayed are allowed to emerge in wire-framed cages 6 × 6 × 6 in. covered with standard, army-issue, mosquito netting; 30 to 100 insects are used per cage, according to the expected spray pick-up. The cages containing the insects are placed in an airtight insulated box which is taken into the spray room 20 min. before starting spraying. This is to avoid adventitious activity due to handling or change of temperature. The spray room, 12 ft. high, has a volume of 750 cu.ft.

When the atmosphere of the spray room has been properly conditioned a determined volume of spray-liquid is sprayed vertically upwards through an 'Aeraspray' gun, model 'Conex G.2', gravity feed, which is clamped in the middle of the floor. The working pressure is 25 lb./sq.in., and the delivery rate is adjusted to 20 ml. of water per minute at a temperature of 30° C.

During spraying the insect cages are kept in the airtight insulated box which contains conditioned air from the breeding room. At some fixed time after spraying the cages are withdrawn carefully, without disturbing the insects, and are placed on an open grid. After the required period of exposure the cages are removed from the spray-room and the insects from each cage are tipped on to a piece of paper and counted, and are then ready for the determination of aluminium. Mosquitoes of the species *A. aegypti* may often be rendered sufficiently inactive by the cool air of the laboratory, but it may be necessary to apply an anaesthetic. It has been found that no significant amount of aluminium is lost by this transference process.

Spray-liquid for water-base sprays. In preparing the spray-liquid, water is replaced by a saturated (40%) solution of aluminium chloride: this is diluted to give a convenient deposit of aluminium for analysis. The maximum permissible concentration is governed by the sensitivity of the spray gun. A concentration of 30% aluminium chloride or less was satisfactory. The diluted solution should be practically neutralized by boiling with successive small quantities of ammonium hydroxide solution, sp.gr. 0·88, until a little aluminium hydroxide persists as a precipitate.

Analytical procedure. The stupefied insects are transferred to a 30 ml. hard glass flask and boiled with nitric acid ('Analar') until no solid matter remains. Oxidation is completed by addition of 0·5 ml. 100 vol. hydrogen peroxide and boiling down, repeating 3 or 4 times. The nitric acid is then evaporated and the aluminium nitrate decomposed, but the residue of alumina must not be overheated. It is dissolved in 0·5 ml. 0·5 N-hydrochloric acid containing

0·1 % hydroquinone to remove residual traces of nitric acid and to prevent later oxidative side reactions.

The acidic solution of aluminium chloride is transferred to a 150 ml. flask and the volume made up to 25 ml. with distilled water. The solution is then buffered with ammonium acetate and the pH adjusted to 7·2 by addition of a solution containing sodium carbonate and potassium cyanide. At this pH the subsequent development of the blue colour is quantitative. The potassium cyanide performs the additional function of suppressing interference from any copper ions. To ensure the stability of the lake when it has been formed, a solution of a protective colloid, 'starch glycerite', is added next and finally the haematoxylin solution.

If the amount of aluminium present is such that the colour produced can be compared with that given by a standard lake containing 0·010 mg. of aluminium a slight blue colour will immediately be developed. A standard lake is prepared at the same time as the lake to be determined and the two are kept for 20 min. to allow the full colour to be developed. They are then compared in a colorimeter previously calibrated for the reaction and the spray pick-up per insect calculated.

The method of analysis outlined above is essentially that of Houghton (1943), with modifications proposed by Strafford & Wyatt (1943), and further altered to adapt it to analysis of spray deposits.

Waterhouse (1947) has summarized the evidence, showing that it is desirable to reduce the 'mean free flight' of flying insects during a biossay to a low level. On the other hand, the reasons for keeping the spray chamber fairly large, say 500–1000 cu.ft. are also indicated in the same paper. It is felt that the arrangement outlined above goes some way towards satisfying both sets of requirements. Otherwise our experimental arrangements are similar to those of David (1946*a*) and Hoskins & Caldwell (1947).

Results

If the insect is allowed to remain undisturbed in an atmosphere containing a non-irritant spray, the amount of spray that will adhere to the insect will depend mainly on the nature of the surface of the insect, being increased by the presence of long or dense patches of hairs, and to a smaller extent by the presence of scales. The amount of spray collected by insects having similar surfaces is, of course, proportional to the surface areas. A factor which is of particular importance in the case of sprays, such as D.D.T. water or oil-case sprays, which lack an irritant or rapidly toxic constituent, is the extent to which the natural activity of the insects causes them to fly and thus to increase the volume of spray accumulated. This natural activity may be standardized to some extent by eliminating a rising gradient of temperature or humidity during spraying, and allowing adequate time for insects to become conditioned to their environment.

As David (1946*b*) points out, the variability of the results of assays with flying insects, using non-irritant insecticides, is greater than when irritant insecticides are used. This increase is related to fluctuations in natural activity between different populations, a source of error which disappears when the insects are stimulated by the pyrethrins.

Choice of responses as criteria of toxicity

The measurement of tolerance was made throughout in terms of the proportionate paralysis after 10 min. exposure. The use of the primary response of paralysis was selected in preference to the more usual secondary response of 24 hr. kill for the following reasons:

First, the magnitude of the primary responses largely determines that of the secondary responses, not only because of the high correlation between various responses in terms of the tolerance of the insect, which would be expected, but also because the dose of pyrethrins acquired by the insect is largely a function of the primary responses.

Secondly, there is good reason to believe from what is known and suspected about the mode of action of the pyrethrins that the processes which precede the two types of responses are materially different. As a consequence the use of a secondary response adds a further series of processes acting over a much longer period than those which precede the primary response. The disturbances which result from the fluctuating biological condition of the insects are thereby unnecessarily accentuated and, in addition, corrections should be applied for control responses.

Thirdly, the values of the primary responses can be directly related to the chemically determined value of the pick-up of pyrethrins, and are therefore more easily interpreted than the secondary responses.

When a definite weight of the pyrethrins, averaged for the population under test, has been accumulated, the activation of the insects increases very rapidly. This weight, which is characteristic of the species of insect at a standard level of tolerance, is defined as the activation threshold, and the corresponding weight for paralysis as the knockdown threshold. These are convenient terms to define narrow ranges of the pyrethrins pick-up over which changes of activity and paralysis are particularly marked and are not meant to represent 'thresholds' in the limiting sense criticized by Horsfall (1945). Strictly, our 'thresholds' are the modal values of the curves representing the distribution of activity and paralysis with increasing pick-up of pyrethrins.

When that concentration of pyrethrins has been reached which will enable the insects to accumulate the activation threshold weight of pyrethrins, natural activity is replaced by forced activity due to the effect of the insecticide on the peripheral nervous system. The spray pick-up/pyrethrum-concentration curve at this point begins to rise along a sigmoid course, reflecting the dosage-response relationship of pyrethrins concentration and flight activity.

As the pick-up is now increasing with increasing pyrethrins concentration, the amount of insecticide collected by the insect ceases to be proportional to the pyrethrins concentration, and rises rapidly until the second of the two controlling thresholds is reached.

Once conditions have been provided in which it is possible for the insects to pick

up a weight of insecticide corresponding to the knockdown threshold, the rapid paralysis of the flying insects is reflected in the sudden drop in the spray pick-up/pyrethrins-concentration curve which is soon reduced to a level dependent only on the inherent time lag of paralysis behind the initial presentation of the toxic spray. As this time lag is not sensibly reduced by increasing the concentration of insecticide, the curve rapidly becomes a straight line parallel to the pyrethrins-concentration axis. The volume of spray represented by this line is only slightly higher than that accumulated by unstimulated insects.

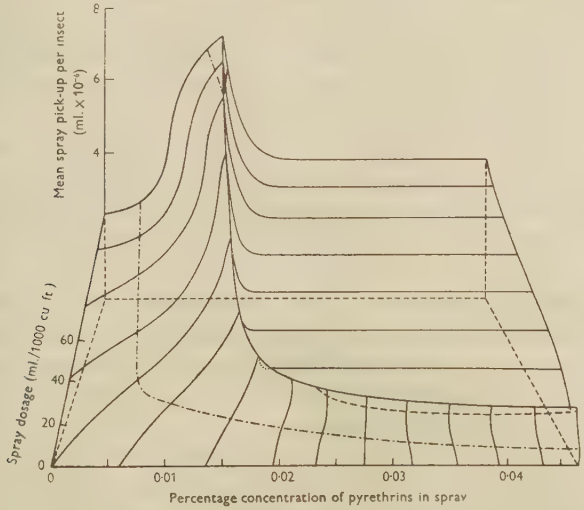


Fig. 1. *Aëdes aegypti* L. Three-dimensional graph of spray pick-up/spray dosage/concentration of pyrethrins. Ridge of graph: knock-down threshold at $6{\cdot}0 \times 10^{-7}$ mg. of pyrethrins picked up; — · — · —, activation threshold at $1{\cdot}2 \times 10^{-7}$ mg. of pyrethrins picked up.

The spray pick-up for unstimulated *A. aegypti* mosquitoes increases approximately linearly with the spray dosage, defined as the volume of liquid sprayed per unit volume of air space. The concentrations of pyrethrins at which the activation and knock-down thresholds become attainable consequently decrease as the spray dosage is increased.

At the higher pyrethrins concentrations when, except for the lowest range of spray dosages, the pyrethrins pick-up exceeds the knock-down threshold weight, the spray pick-up/spray-dosage curves are of sigmoid shape, with the flatter part of the curve covering the usual range of spray dosages. This observation may be related to the findings of Murray (1940) that, in the Peet-Grady method of insecticide testing against flying insects, especially houseflies, changes of 50% in measuring the spray dosage do not cause the mean spray pick-up per insect to differ to an extent which exceeds the standard error of the mean at the correct spray dosage (see also Fig. 7).

Consideration of the changes in spray pick-up occurring in the area between the two pyrethrins thresholds (and slightly beyond the knockdown threshold) shows that, in this critical region which covers the knockdown range most used in biological assays, an increase in spray dosage may either increase or decrease the spray pick-up associated with it. It is implicit in almost all previous discussions concerning the dosage response relationships of insects flying in sprays containing pyrethrins that the pick-up of pyrethrins on the insect is directly proportional, not only to the spray dosage, but also to the concentration of pyrethrins in the spray. Over the important range of responses considered neither assumption is valid even as an approximation. It is hoped that further work utilizing the actual pyrethrum pick-up on the insect as the criterion of insecticide dosage and the knockdown dependent on this as the criterion of response will serve to elucidate the apparent anomalies displayed in the conventionally sigmoid dosage response curve.

As the pyrethrins concentration is increased, at any given spray dosage, the mean time taken for the insects to become paralysed is progressively reduced until that concentration is reached which enables the insects to accumulate the knockdown threshold dose within the given exposure period. At pyrethrins concentrations higher than this, the rate of paralysis and also the total volume of spray liquid accumulated, become independent of the concentration. Under these conditions, the minimum time of paralysis is approximately 2·5 min., and is determined solely by the time taken for the pyrethrins to diffuse through the insect cuticle.

This limiting time of diffusion is one of the parameters of the Fick diffusion equation which can be used to describe the transfer of the pyrethrins molecules to their site of action in the insect. Another parameter is the diffusion coefficient of the pyrethrins molecules, which may be estimated from a knowledge of their mean molecule weights, and the physical properties of the insect epicuticle. Since, under the conditions mentioned above, the rate of paralysis is independent of the pyrethrins concentration, the appropriate bounding condition for the Fick equation is that of diffusion from an infinite reservoir of fixed concentration across a membrane. The thickness of the barrier thus presented to the pyrethrins may then be estimated as probably about 0·20 μ thick, and certainly not more than 1·0 μ thick, being the only 'unknown' parameter in the equation. Allowance for the time lag between presentation and pick-up of spray, would reduce this estimate still further.

These figures, which agree very well with the more direct measurements of the thickness of the waxy epicuticle 0·1–0·4 μ by Beament (1946), indicate that the site of action of the pyrethrins is very close to the surface of the insect, i.e. probably in the endings of the peripheral nervous system. So far as the test systems discussed in this paper are concerned, any mechanism for the toxic action of the pyrethrins which involves transfer of the insecticides to the central nervous system can be ruled out.

The sharp onset of paralysis, once the knockdown threshold is attained, is illustrated by the change in the curves representing the rate of pick-up of spray by

the flying insects. Whereas the points measured below the knockdown threshold concentration, at a given spray dosage, lie on a sigmoid curve characteristic of weak drug action, those measured above the threshold fall on what approximates to an exponential curve, such as is more usual with powerfully acting drugs (Clarke 1933).

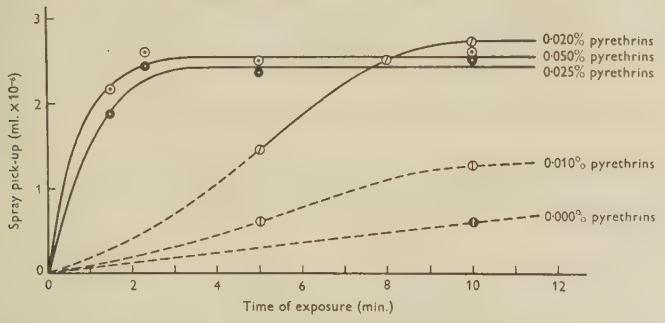


Fig. 2. Variation of spray pick-up on *Aëdes aegypti* L. with time of exposure to spray. Nominal spray dosage, 13 ml. per 1000 cu.ft.; temperature, 30° C.; Relative humidity, 100%.

The effect of an increase of spray dosage on the rate of pick-up at maximum flight activity, is to increase the steepness of the sigmoid curves representing the pick-up against period of exposure.

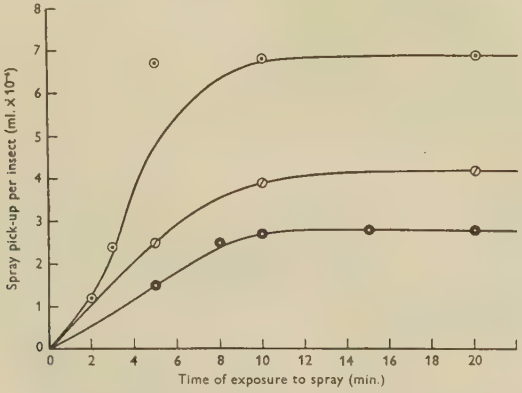


Fig. 3. Variation of spray pick-up on *Aëdes aegypti* L. at maximum activity with time of exposure to spray.

Spray dosages: —⊙—⊙— 66 ml./1000 cu.ft.; —⊘—⊘— 40 ml./1000 cu. ft.; —●—●— 13 ml./1000 cu.ft.

The relatively high spray pick-up at maximum activation when low spray dosages are used, coupled with the relatively small increases which result from large increases in the normal range of spray dosages, for any particular species, have led us to suppose

that the absolute efficiency of the spray must decrease over this range, and suggest that this variation of efficiency should be a useful indication of the behaviour of the spray under varying conditions.

When a given volume of liquid is dispersed in the form of a mist of droplets of varying particle sizes, some of the droplets will settle too quickly whilst others will be too small to wet the insect. Consequently, the volume dispersed represented by the droplets which, for biological reasons, are available to be picked up by the insect, may be considered to represent the effective spray dosage. The ratio $\frac{\text{effective dosage}}{\text{nominal dosage}}$ may be considered a measure of the efficiency of the spray, provided that it is borne in mind that other considerations not included in the definition may affect the ultimate practical usefulness of the spray.

The special case where the efficiency of the aqueous spray is diminished by reducing the humidity of the atmosphere into which it is sprayed has been studied because of its practical importance. Reduction of the humidity of the air increases by evaporation the number of droplets which are too small to be effective. As the insect's behaviour does not appear to be influenced by the change of humidity, at least during the periods used for conditioning, the result of spraying an aqueous spray into an unsaturated atmosphere is equivalent to a reduction in the nominal spray dosage at saturation humidity.

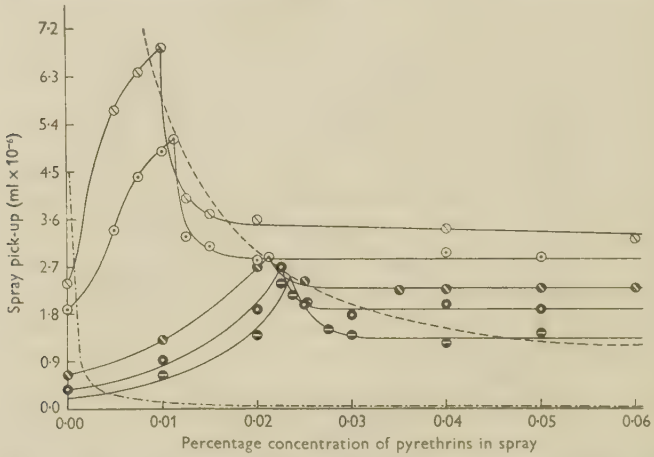


Fig. 4. Variation of spray pick-up by *Aëdes aegypti* L. with humidity at various spray dosages.
—⊘—⊘— Spray dosage 66 ml./1000 cu.ft. at 100 % relative humidity.
—⊙—⊙— Spray dosage 66 ml./1000 cu.ft. at 70 % relative humidity.
—⊘—⊘— Spray dosage 13 ml./1000 cu.ft. at 100 % relative humidity.
—⊙—⊙— Spray dosage 13 ml./1000 cu.ft. at 70 % relative humidity.
—⊙—⊙— Spray dosage 13 ml./1000 cu.ft. at 40 % relative humidity.
– – – – – – Knockdown threshold of $6{\cdot}0 \times 10^{-7}$ mg. pyrethrins pick-up.
– · – · – · Activation threshold of $1{\cdot}2 \times 10^{-7}$ mg. pyrethrins pick-up.

This reduction of nominal spray dosage may be calculated from the shift in the knockdown pyrethrins concentration, produced by lowering the humidity.

TABLE 1

Dosages in ml./1000 cu.ft.

Nominal dosage (100% R.H.)	Equivalent dosage (70% R.H.)	Equivalent dosage (40% R.H.)
66	55	—
13	10	7·5

It should be noted that the effective dosage is less than the equivalent dosage, and although it is probably related to the latter much as it is to the nominal spray dosage, the exact relationship is unknown.

The theoretical aspects of spray efficiency and allied topics in relation to insecticidal sprays will be discussed in a later paper of this series.

WATER-BASE SPRAYS AND SOME FLYING INSECTS OTHER THAN *AËDES AEGYPTI* L.

Diptera (*Nematocera*): Culex pipiens *L.*

The general features exhibited by the set of spray pick-up/toxicant-concentration curves for *Aëdes aegypti* at various spray dosages are repeated by those obtained with *Culex pipiens*.

Whereas the area below the hyperbola representing the activation threshold is all but suppressed with *Aëdes aegypti*, it is clearly shown for *Culex pipiens*, for which the activation and knockdown threshold values are considerably higher. An interesting feature in this area is the sigmoid character of the spray pick-up/spray-dosage curve at zero pyrethrins concentration, which, in the case of *Aëdes aegypti* cannot be demonstrated.

Although the spray efficiency curves for *Culex pipiens* are exactly analogous to those for *Aëdes aegypti* there does not seem to be the same tendency for the knockdown threshold value to become abnormal at high spray dosages. On the other hand, precise measurements of the reactions of the insect under critical conditions is rendered difficult since collection of egg rafts results in the rearing of variable strains even if a standard rearing technique is adopted.

Diptera (*Cyclorrhapha*): Musca domestica *L.*

The housefly, *Musca domestica* L. is important as the standard test insect in the majority of insecticide testing systems using flying insects. In such systems the sprays are generally applied to the assay of oil-base insecticides and investigation of the reactions of *M. domestica* in water-base sprays, though probably analogous, are unlikely to give a close quantitative correspondence with those obtained from oil-base sprays.

House-flies show the same type of spray pick-up curves as the other insects investigated. The form of the curve is that generally found for insects with fairly low unactivated spray pick-up, i.e. it has a long flat portion below the activation

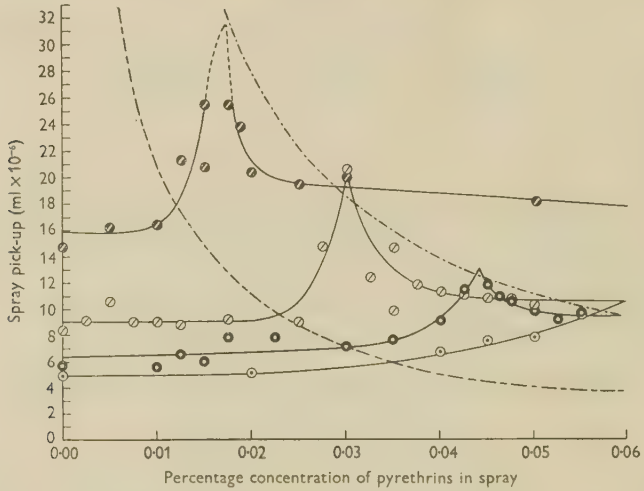


Fig. 5. Pick-up of spray by *Culex pipiens* L.

Aqueous emulsion at 33° C. and 100 % relative humidity. Spray dosage:
66 ml./1000 cu.ft. 40 ml./1000 cu.ft.
13 ml./1000 cu.ft. 6·5 ml./1000 cu.ft.
Activation threshold. Knockdown threshold.

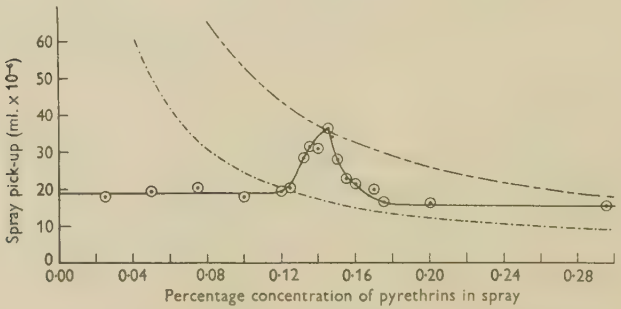


Fig. 6. Pick-up of spray by *Musca domestica* L. Knockdown threshold; Activation threshold.
Spray pick-up at 33° C., 100% relative humidity and spray dosage of 40 ml./1000 cu. ft.

threshold. It is important to note that the physiological condition of the insect affects not only its resistance in terms of the threshold toxicity values for pyrethrins but also of the position of the peak in the spray pick-up/pyrethrins-concentration

curves, which is displaced along the pyrethrins concentration axis. This shift in the peak renders the actual dose received by the insect largely dependent on its resistance, a variability quite distinct from, and independent of, the fact that the spray pick-up on individual insects is distributed about the mean with a high standard deviation, a point proved for houseflies in oil-based sprays by Murray (1940).

The 50% response range recommended on account of the increased sensitivity of dosage response relationships in that region is now generally adopted for bioassay work. Unfortunately, this region includes that level of paralysis usually associated with the onset of the knockdown threshold pyrethrins values, irrespective of the spray dosage at which the measurements are made.

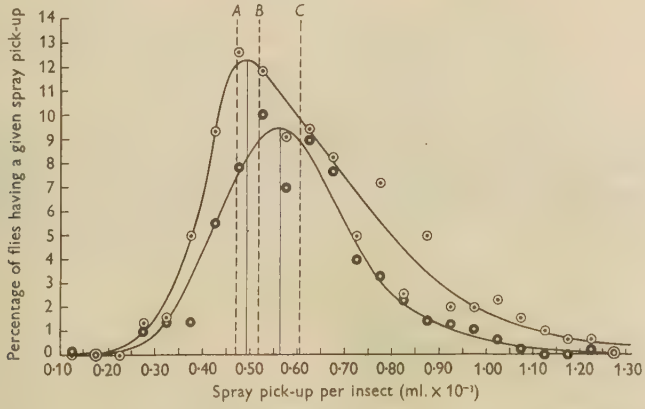


Fig. 7. Distribution of spray pick-up by *Musca domestica* L. in the peet-grady test. Data from Murray (1940). Volume of chamber = 6′ × 6′ × 6′ = 216 cu.ft. Concentration of pyrethrins = 0·10 %. Spray dosage = 111 ml./1000 cu.ft. —⊙—⊙— 304 female flies. —●—●— 532 male flies. *A*, Mean spray pick-up for spray dosage of 12 ml./1000 cu.ft. *B*, Mean spray pick-up for spray dosage of 24 ml./100 cu.ft. *C*, Mean spray pick-up for spray dosage of 36 ml./100 cu.ft.

The difficulty applies to all the flying insect systems which we have studied and is probably general. The problem is less serious with the smaller insects such as mosquitoes than with the larger insects such as the houseflies, since the variations caused by it are in the former case small compared with the experimental error. There is, for aero-dynamic reasons, a marked correlation between the weight of an insect and the volume of air swept out by its wings. The latter quantity is connected by Knudsen's Cosine Law with the number of spray droplets collected on the wings.

The general problem of the impaction of droplets on insects has been investigated by Kennedy, Ainsworth & Toms (1948) and the physical properties of oil-based sprays have been extensively studied by David (1946*b*) and David & Bracey (1946). These aspects of the interaction of insecticidal sprays and flying insects will not be further discussed here.

As there is also a well-known relationship between the weight of an insect and its resistance, it follows that a linear relationship should be obtainable between the spray pick-up and the two pyrethrins thresholds, activation and paralysis.

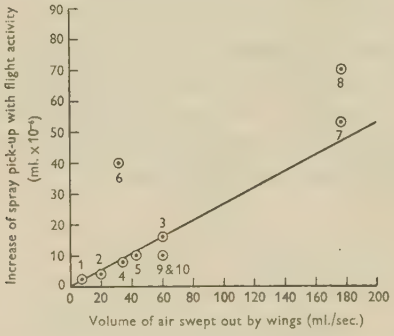


Fig. 8. Variation of spray pick-up with flight activity. 1, *Aëdes aegypti* L.; 2, *Culex pipiens* L.; 3, *Coccinella septempunctata* L.; 4, *Rhagonycha fulva* Scop., males; 5, *Rhagonycha fulva* Scop., females; 6, *Musca domestica* L.; 7, *Vespula germanica* Fab., females; 8, *Vespula vulgaris* L., females; 9, *Vespula germanica* Fab., workers; 10, *Vespula vulgaris* L., workers.

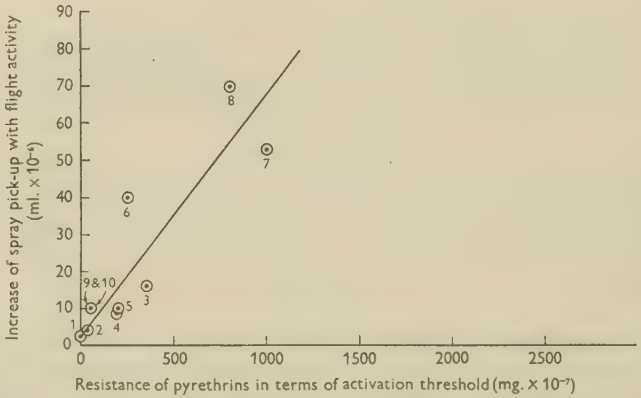


Fig. 9. Variation of spray pick-up and resistance in terms of activation threshold. 1, *Aëdes aegypti* L.; 2, *Culex pipiens* L.; 3, *Coccinella septempunctata* L.; 4, *Rhagonycha fulva* Scop., males; 5, *Rhagonycha fulva* Scop., females; 6, *Musca domestica* L.; 7, *Vespula germanica* Fab., females; 8, *Vespula vulgaris* L., females; 9, *Vespula germanica* Fab., workers; 10, *Vespula vulgaris* L., workers.

The practical significance of these two relationships is that the general form of the spray pick-up curves for any flying insect can be drawn, in part quantitatively, from a knowledge of the body weight, or the dimensions of its wings and frequency of its wingbeat.

The most important influence on the form of the spray pick-up curve, other than the volume of air swept out by the wings, is the unstimulated spray pick-up, which depends on the natural 'hairiness' of the insect, and its normal tendency to flight in a non-irritant spray.

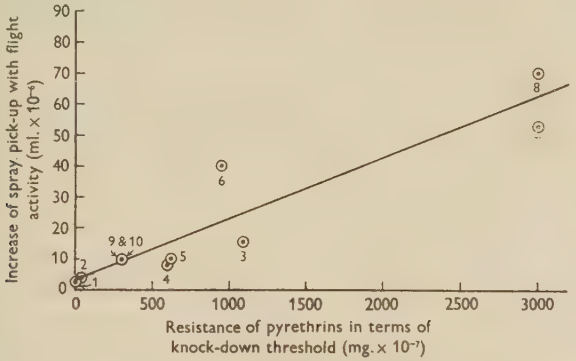


Fig. 10. Variation of spray pick-up and resistance in terms of knockdown threshold. 1, *Aëdes aegypti* L.; 2, *Culex pipiens* L.; 3, *Coccinella septempunctata* L.; 4, *Rhagonycha fulva* Scop., males; 5, *Rhagonycha fulva* Scop., females; 6, *Musca domestica* L.; 7, *Vespula germanica* Fab., females; 8, *Vespula vulgaris* L., females; 9, *Vespula germanica* Fab., workers; 10, *Vespula vulgaris* L., workers.

A point of some interest is that the ratio $\frac{\log_e (\text{knockdown threshold})}{\log_e (\text{activation threshold})}$ is approximately constant, indicating that the mechanism of action of the pyrethrins is similar in all the cases studied.

TABLE 2

Species	$\frac{\log_e (\text{knockdown threshold})}{\log_e (\text{activation threshold})}$
Aëdes aegypti L.	1·65
Culex pipiens L.	1·19
Coccinella septempunctata L.	1·14
Rhagonycha fulva Scop., ♂	1·15
Rhagonycha fulva Scop., ♀	1·15
Musca domestica L.	1·17
Vespula germanica Fab., ♀	1·12
Vespula germanica Fab., ☿	1·29
Vespula vulgaris L., ♀	1·15
Vespula vulgaris L., ☿	1·29

The difference between the value of the ratio for *Aëdes aegypti* and that for the other insects is due to the fact that the ratio for the former can be deduced by a number of different methods, whereas the figures for other insects have only been estimated from their spray pick-up/pyrethrins-concentration curves. This method gives results which, as Table 2 shows, are consistent among themselves, but the figure for *A. aegypti* is the more accurate.

It may be noted here that the value for the activation threshold for *A. aegypti* given by Page, Stringer & Blackith (1946) should read $1{\cdot}20 \times 10^{-7}$ mg. and not $0{\cdot}12 \times 10^{-7}$ mg.

The activation and paralysis thresholds for *A. aegypti* are about half the corresponding values which may be deduced from the figures given by David & Bracey (1946). The latter figures were, however, stated to be only approximate.

Coleoptera (*Clavicornia*): Coccinella septempunctata *L.*

The 'seven-spotted ladybirds' exhibit considerable resistance to pyrethrins but are, nevertheless, stimulated eventually to flight activity by them. The insects attempt to clean even harmless spray droplets from the head and thorax with the prothoracic legs. When pyrethrins are present in the spray in sufficient concentration to compensate for the relatively low spray pick-up on the smooth compact cuticle, the insect cocks its elytra and unfolds its wings, which it beats intermittently for short periods. Actual flight is achieved but rarely in the latter stages of intoxication, as the insects tend to move backwards and, falling on the tripod formed by the head and cocked elytra, are free to continue the beating of their wings.

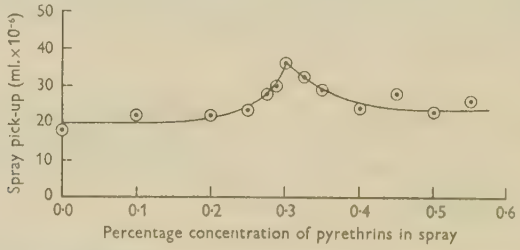


Fig. 11. Pick-up of spray by *Coccinella septempunctata* L. Spray dosage, 40 ml. 1000 cu.ft.; relative humidity, 100%; temperature, 30° C.; aqueous spray.

The spray pick-up curve for *C. septempunctata* shows that there is an unusually wide range of pyrethrins concentrations below the activation threshold which do not affect the insect. This fact arises partly from the low spray pick-up and partly from the relatively high body weight of the insects.

Coleoptera (*Malacodermata*): Rhagonycha fulva, *Scop.*

The considerable sexual dimorphism exhibited by 'soldier beetles' makes a comparison of the spray pick-up/pyrethrins-concentration curves of the two sexes of interest, especially as the wing areas are very similar, although the abdomen of the female projects beyond the length of the elytra, whereas that of the male is scarcely visible from above. It is necessary to collect the insects whilst swarming before mating, as copulation causes changes in the male usually resulting in early death. The sexes were separated during collection and the males preserved in a cage for

24 hr. to eliminate, as far as possible, those whose resistance had been reduced by mating.

The spray pick-up curve for the male insects runs higher than that for the females, below the knockdown threshold, probably on account of the greater natural activity of the males, but beyond the knockdown threshold the two curves appear to run together.

The knockdown and activation thresholds of the two sexes are similar, despite the differences in spray pick-up with the result that the two sexes have differing knockdown threshold pyrethrins concentrations.

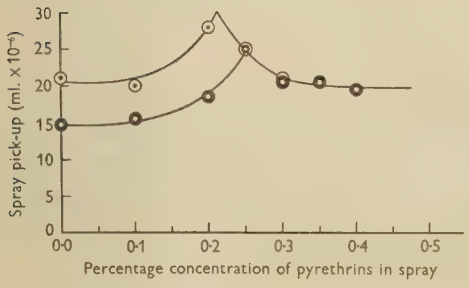


Fig. 12

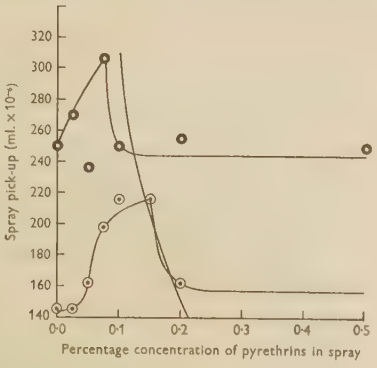


Fig. 13

Fig. 12. Pick-up of spray by *Rhagonycha fulva* Scop. Spray dosage, 40 ml./1000 cu.ft.; —⊙—⊙—, male insects; —●—●—, female insects; aqueous spray; relative humidity, 100%; temperature, 30° C.

Fig. 13. Comparison of spray pick-up of *Vespula* spp. flying in cages. —⊙—⊙—, *Vespula vulgaris* L. (queens); —●—●—, *V. germanica* Fab. (queens); ———, knockdown threshold 3000×10^{-7} mg. pyrethrins. Spray dosage 40 ml./1000 cu.ft.

Hymenoptera (*Aculeata*): Vespula germanica *Fabr. and* V. vulgaris *L.*

The behaviour of wasps, exposed to pyrethrum-bearing sprays, shows interesting modifications on account of the relatively high spray pick-up on the extensive hair-covered regions of the body. Whilst it is difficult to obtain reproducible results with worker wasps, on account of their differing ages and occupations, the queens of any one nest, excepting the functional queen, are of similar ages and if the nest be taken just prior to the immature queens' mating flights, reasonably reliable information may be obtained even from the reaction of single insects.

A point of some interest is the considerable difference in the pick-up, not due to stimulated flight activity, between the queens of the two species studied. Whilst part of this difference is probably to be related to specific differences in the number or structure of the hairs covering the insects, such differences are not obvious on

inspection, and the greater natural 'aggressiveness' of *V. germanica* may be a contributing factor.

The distinction between the species may be extended to the workers, as shown in Fig. 14.

The high spray pick-up on the stationary insect causes the peak activity concentration of pyrethrins to be exceptionally low for so large an insect. This fact, coupled with the variations in the resistance and behaviour of the worker wasps, depending on their previous history, tends to make the delineation of the peak uncertain. There is, however, no reason to believe that the course of the spray pick-up/toxicant concentration curves for these insects does not follow the type set by *Aëdes aegypti*.

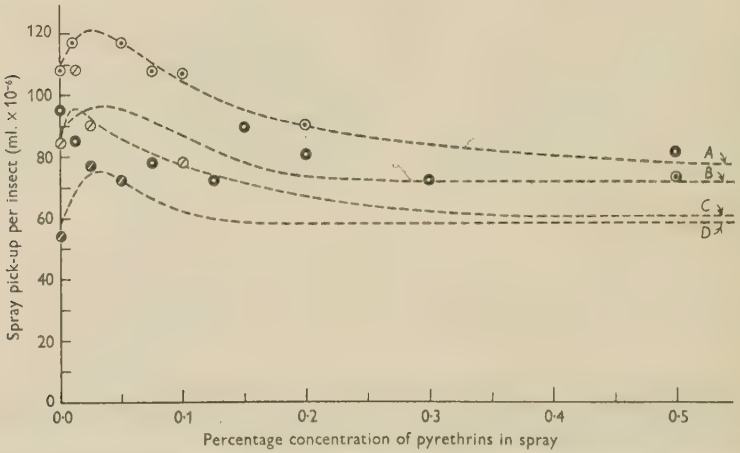


Fig. 14. Pick-up of spray by *Vespula germanica* Fab. and *V. vulgaris* L. (workers).

Vespula germanica Fab. A —⊙—⊙— 40 ml. per 1000 cu.ft.
B —●—●— 13 ,, ,,
V. vulgaris L. C —⊘—⊘— 40 ,, ,,
D —◐—◐— 13 ,, ,,

The onset of the knockdown threshold pyrethrins concentration, as measured by the fall in the spray pick-up/pyrethrins-concentration curve, may be compared with the curve obtained by plotting the time taken to paralyse the insect at a given pyrethrins concentration against that concentration.

Owing to the high spray pick-up attained by queen wasps, and their comparatively stable level of resistance, the reactions of single insects suspended in mid-air may be studied and compared with those of the caged insects.

When the insect is lifted off the substrate it automatically commences the motion of flight, which it maintains in general until exhaustion or paralysis supervene. The amount of spray picked up, at zero pyrethrins concentration, is then the saturation

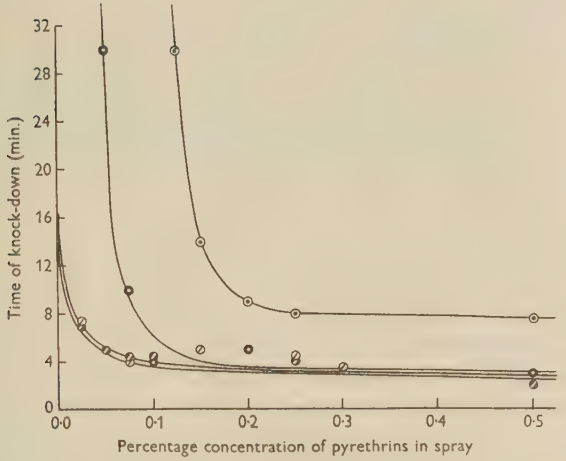


Fig. 15. Rate of action of pyrethrins on *Vespula germanica* Fab. (queens).

—o—o— Flying in cages 40 ml. per 1000 cu.ft.
-⊘---⊘- Free flying (suspended) ,, ,, ,,
—⊙—⊙— Flying in cages 13 ml. per 1000 cu.ft.
—⊘—⊘— Free flying (suspended) ,, ,, ,,

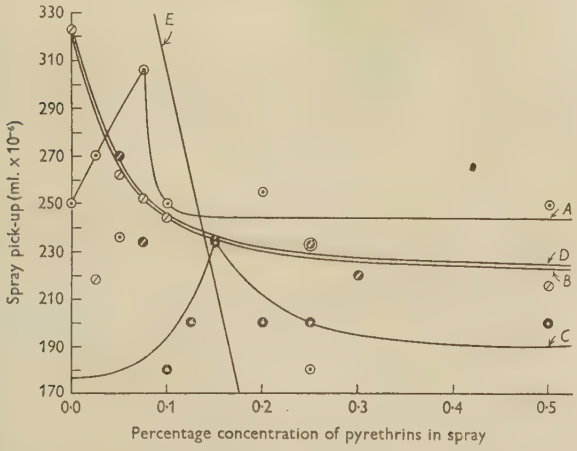


Fig. 16. Pick-up of spray by free flying and caged *Vespula germanica* Fab. (queens).

A —⊙—⊙— Flying in cages 40 ml. per 1000 cu.ft.
B —⊘—⊘— Free flying (suspended) ,, ,,
C —o—o— Flying in cages 13 ml. per 1000 cu.ft.
D —⊘—⊘— Free flying (suspended) ,, ,,
E ———— Knockdown threshold 3000×10^{-7} mg. pyrethrins.

value for the insect in flight, as it attains its limiting value well before the expiration of the standard 10 min. exposure period.

As in the case of *A. aegypti*, the saturation pick-up is not much above the value of the spray pick-up at full activation for the higher spray dosages used, and excess spray liquid picked up is thrown centrifugally from the wing-tips.

When female or worker wasps are suspended in the spray, the rate at which they accumulate the spray liquid is so great that the weight of the pyrethrins required to produce paralysis is rapidly attained, even when the pyrethrins concentration is quite low. The 'peak' in the spray pick-up/pyrethrins-concentration curves therefore occur at much lower concentrations than might be expected for a relatively large insect. Since, in addition, the spray pick-up at zero pyrethrins concentration is very high, the 'peak' is ill-defined, as will be seen from Fig. 14.

The apparent identity of the spray pick-up curves for 40 and 13 ml./1000 cu.ft. for suspended queens of *Vespula germanica*, is paralleled by the apparent identity of the time of knockdown/pyrethrins-concentration curves for those two spray dosages (Figs. 14 and 15). This merging of the two curves, distinctly separated for the cage insects, is probably due to the rate of pick-up of spray under what amounts to peak activation conditions approaching the rate of action of pyrethrins at the lower insecticide concentrations.

At the knockdown pyrethrins concentration the spray pick-up curves for the suspended and caged insects tend to run parallel to one another, and this is also shown by the curves representing the times of paralysis with increasing pyrethrins concentration.

We are indebted to Dr G. Fraenkel for demonstrating to us his technique for suspending flying insects, and to Mr R. C. B. Hartland-Rowe for much valuable assistance in the breeding and collecting of the various test insects used.

REFERENCES

Beament, J. W. L. (1946). The water-proofing process in eggs of *Rhodnius prolixus*, Stähl. *Proc. Roy. Soc.* B, **133**, 407.

Clarke, A. J. (1933). *The Mode of Action of Drugs on Cells.* London: Arnold and Co.

David, W. A. L. (1946*a*). Factors influencing the interaction of insecticidal mists and flying insects. Part I. The design of a spray testing chamber and some of its properties. *Bull. ent. Res.* **36**, 373.

David, W. A. L. (1946*b*). Factors influencing the interaction of insecticidal mists and flying insects. Part II. The production and behaviour of kerosene-base insecticidal sprays, mists and their relation to flying insects. *Bull. ent. Res.* **37**, 1.

David, W. A. L. & Bracey, P. (1946). Factors influencing the interaction of insecticidal mists and flying insects. Part III. Biological factors. *Bull. ent. Res.* **37**, 177.

Horsfall, J. G. (1945). *Fungicides and their Action.* Waltham, Mass.: Chronica Botanica, Co.

Hoskins, W. M. & Caldwell, A. H., Jr. (1947). The design and use of a small spray chamber. *Soap*, **22**, 143.

Houghton, G. U. (1943). The determination of small amounts of aluminium in water by means of haematoxylin. *Analyst*, **68**, 208.

KENNEDY, J. S., AINSWORTH, M. & TOMS, B. A. (1948). *Laboratory Studies on the Spraying of Locusts at Rest and in Flight.* London: Anti-Locust Research Centre, Bull. no. 2.

MURRAY, C. A. (1940). A fundamental error in the Peet-Grady method. *Soap*, **16**, 111.

PAGE, A. B. P., STRINGER, A. & BLACKITH, R. E. (1946). Investigation of insecticidal sprays. *Nature, Lond.*, **157**, 80.

STRAFFORD, N. & WYATT, P. F. (1943). The separation and determination of very small amounts of aluminium and iron in water. *Analyst*, **68**, 319.

WATERHOUSE, D. F. (1947). An examination of the Peet-Grady method for the evaluation of household sprays. *Bull. Coun. sci. industr. Res. Aust.* no. 216.

(*Received* 30 *October* 1948)

BIOASSAY SYSTEMS FOR THE PYRETHRINS

II. THE MODE OF ACTION OF PYRETHRUM SYNERGISTS

By A. B. P. PAGE and R. E. BLACKITH

Imperial College of Science and Technology Field Station, Silwood Park, Sunninghill, Berks

(With 1 Text-figure)

The components of 'synergistic action' are reviewed in the light of recent work. It has been shown that for sesamin and isobutylundecylenamide at least, synergistic action remains after physical effects are eliminated. The toxicity of the pyrethrins is increased by a factor of three when up to equimolecular proportions of the above synergists are added, further additions having no effect. It is suggested that a surface complex between synergist and insecticide is formed at the peripheral nerve sheath interfaces, resulting in the reorientation of the pyrethrins molecules to give a more efficient discharge of the resting potential at the interface.

The synergistic action of sesame oil and isobutylundecylenamide when added to solutions of the pyrethrins in oil has been known for some years. The active ingredient of sesame oil has been identified as sesamin, with other and less effective ingredients such as sesamolin and the optical isomers of sesamin also present (Haller, Laforge & Sullivan, 1942*a*,*b*; Haller, McGovran, Goodhue & Sullivan, 1942). It is, however, still customary to work with the crude oil at about 5% concentration. This crude oil has very variable activating properties, depending mainly on the sesamin content, usually about 1·0% w/v.

The components of 'synergism'

David & Bracey (1944) showed that when sesame oil is incorporated in a preparation containing pyrethrum, the solvent of which is volatile, i.e. an aerosol, a large part of the synergistic action consists of a mechanical increase in the size of the droplets, the sesame oil being non-volatile. The larger droplets impact more readily on the wings of the flying insects, thus increasing the effectiveness of the spray, since if the droplets are too small they are carried round the wings and body of the insects in the airflow. Clearly, if this were the only effect of the synergist, sesame oil could be replaced by any non-volatile oil, and indeed David & Bracey were able to obtain this increase in particle size using a heavy lubricating oil. The efficacy of isobutylundecylenamide in oil-base pyrethrum sprays has doubtless been attended by the same effect, since this substance also is non-volatile and concentrations of 2–4% are commonly used, and even 10% has been recorded in the literature.

Parkin & Green (1944), showed, however, that pyrethrum sprays containing sesame oil were consistently more effective than those containing the same volume

of heavy lubricating oil, and emphasized the important distinction between the residual oil effect in aerosol-type sprays and other synergistic effects. These authors were also able to show that sesamin did not necessarily depend for its action on retarding the rate of paralysis of flying *Musca domestica*, contrary to the findings of David & Bracey (1944) using *Aëdes aegypti*.

Hartzell and his co-workers (Hartzell & Scudder, 1942; Hartzell, 1945; Hartzell & Wexler, 1946) have carried out an extensive histological examination of the nervous systems of insects poisoned by the pyrethrins with and without various added synergists. Their studies were, however, carried out on the constituent parts of the central nervous systems of the insects, notably those of *Musca domestica*, and it has been shown (Page, Stringer & Blackith, 1949) that not only do the pyrethrins act primarily on the peripheral nervous system of the insects, but that the site of action is only a few thousandths of a millimetre from the external surface of the insects. This conclusion holds not only for the particular test system employed, but is likely to be true for most conditions of practical importance. Hartzell *et al.* took precautions to eliminate strictly post-mortem degenerative effects in their specimens, but Richards (1941) has shown that changes in the nerve cells similar to those found by Hartzell *et al.* can be simulated by anaeroxia, which is quite likely to be a secondary effect of paralysis by the pyrethrins.

The symptoms of nerve degeneration obtained by Hartzell *et al.* with the synergists alone, were produced only under conditions, concentration, solvent, etc., which bear little relation to those under which the true synergistic effect is clearly shown, and the present authors do not think that the effects obtained throw any light on the mode of action of the synergists in the test systems under discussion.

Modifications of the test conditions may accentuate the contribution of any one of the above subsidiary physical and biological components of synergistic action, but clearly certain substances can increase the toxicity of the pyrethrins in a manner not wholly explicable in terms of these components.

Results

The residual oil effect may be completely eliminated by using the pure compounds, sesamin and isobutylundecylenamide (I.N. 930), at concentrations of 0·005–0·05%, this being the sensitive range for insects such as *Aëdes aegypti*. We have adopted this method, using, in addition, the synergists and the pyrethrins emulsified in a water-base spray liquid as described in Page *et al.* (1949). The method of determining the quantities of the insecticide and synergist actually picked up by the insects in flight is also described in the same paper.

The mean weight of the pyrethrins required to paralyse a standard population of *A. aegypti* mosquitoes was determined in the presence of varying proportions of the synergists. Both sesamin and isobutylundecylenamide were used, the results being identical for both substances.

The most important result obtained by this technique is that the addition of the

synergists reduced the mean weight of the pyrethrins required to paralyse each insect from 6·0 to $2{\cdot}0 \times 10^{-7}$ mg. This reduction was proportional to the mol fraction of the synergists, until these were present in equimolecular proportions with the pyrethrins. Once this 1:1 ratio had been attained, no further reduction in the knock-down threshold weight of the pyrethrins could be achieved, further additions of the synergist having no effect at all.

The range of concentrations up to 0·05% w/v of the pyrethrins was studied. It will be seen from the discussion of the test system in Page *et al.* (1949) that higher

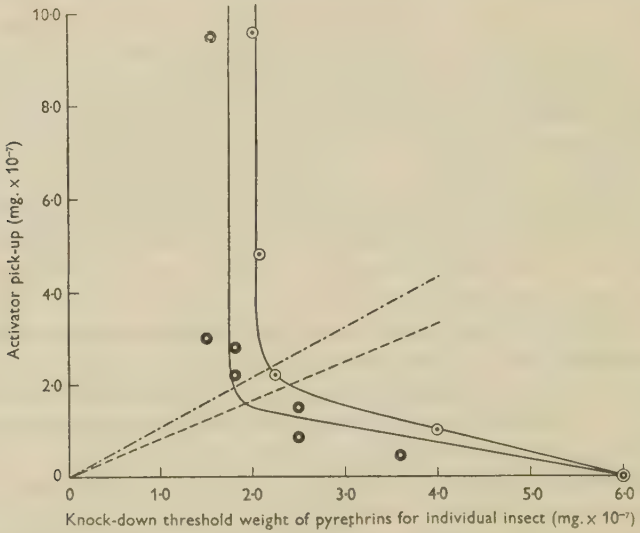


Fig. 1. Activation of pyrethrins in water base sprays. Test insect *Aëdes aegypti* L.:

$-\cdot-\cdot-\cdot-$ $\frac{\text{mol. wt. sesamin}}{\text{mol. wt. pyrethrins}} = 1{\cdot}08.$ $- - - - -$ $\frac{\text{mol. wt. I.N. 930}}{\text{mol. wt. pyrethrins}} = 0{\cdot}73.$

—⊙—⊙— Activation by sesamin. —o—o— Activation by I.N. 930.

concentrations of insecticide are not of interest under these conditions. At no concentration from 0·001 to 1·00% were the synergists toxic or irritant when applied without the pyrethrins, neither did their presence in the spray liquid affect the spray pick-up of the unstimulated insects as compared with the quantity picked up by them from the spray liquid alone.

Discussion: the mechanism of synergistic action

It is clear that we are dealing here with pure synergistic action identical for both substances, and characterized by maximum effectiveness in the presence of equal numbers of the synergist and pyrethrins molecules. It seems reasonable to suppose that a loose molecular complex is formed between the pyrethrins and the synergist

molecules, and that this complex is almost exactly three times as toxic as the pyrethrins alone.

David & Bracey (1944) suggested that the sesamin and isobutylundecylenamide delayed the rate of paralysis of the insects flying in pyrethrum sprays, thus enabling the insects to accumulate more of the insecticide and to respond with a higher 24 hr. mortality.

The opposite explanation has also been suggested (Munro, 1942), namely, that the synergists assist the penetration of the insecticides through the insect cuticle, by increasing the permeability of the latter.

Such hypotheses could not previously be tested as conditions had not been established under which it was certain that the limiting time of diffusion of the pyrethrins through the insect cuticle determined the rate of action of the pyrethrins, without complications due to the variable viscosity of the oil base spray fluids in the presence of different proportions of the synergists. These conditions are satisfied by the higher dosage region of the *A. aegypti*—pyrethrins—aqueous spray system, as discussed in Page *et al.* (1949). It can definitely be stated that, under such conditions, the presence of the synergists has no effect whatever on the rate of paralysis of the flying insects by the pyrethrins, neither is the volume of spray liquid picked up by the insects in any way influenced. Both the hypotheses mentioned above are therefore eliminated.

Preliminary results have been obtained with the two synergists under discussion in the system in which the pyrethrins in an oil-base solution are tested against crawling insects, in this case *Calandra granaria*. These results indicate that in this system also the synergists have the same mode of action as in the flying insect assay. Both sesamin and isobutylundecylenamide increase the toxicity of the pyrethrins by a factor of about three, as in the other case, and there are indications that piperonyl butoxide has a similar effect.

Thus the mode of action of the 1:1 synergist-pyrethrins mixture is probably the same whether it is dissolved in an aqueous or non-aqueous solvent. There is some difficulty however in visualizing any chemical reaction with the pyrethrins common to both sesamin and isobutylundecylenamide. These facts taken together suggest that complex formation may take place at an interface within the insect cuticle, since such surface complexes are well known to be formed, stoichiometrically, between complicated molecules which would not combine in the bulk phase.

It will be noted (Page *et al.* 1949, fig. 4 and 1946, fig. 1) that the weight of pyrethrins at higher concentrations required to paralyse a population of flying insects is less than the knockdown threshold determined at the minimum concentration of pyrethrins with which paralysis is attainable within the given period of exposure. It appears that the pyrethrins have a greater absolute toxicity when presented to the insects at rather higher concentrations than those required to produce the selected response. This increase in toxicity is a general one for all the insect species studied, as will be seen from the graphs showing the variation of spray pick-up with increasing pyrethrins concentration (Page *et al.* 1949).

Richards (1943) has suggested that insecticides become concentrated at lipid-protein interfaces of nerve sheaths. We suggest that in such situations, pyrethrins molecules are orientated differently when presented in concentrations more than sufficient to cover, by adsorption, the interfacial area. It is possible that the adsorption of the insecticide molecules results in the resting potential of the nerve interface between lipoid and protein layers being discharged. Such a discharge mechanism might be facilitated by reorientation following close-packing of the adsorbed molecules. The increase in toxicity of the insecticide, over the concentration range discussed above, is accompanied by a secondary peak, over the same range, in the paralysis-concentration graph for *Aëdes aegypti*. The treatment of this type of curve is made difficult by the fact that at present it is not possible to combine the results from more than one population on account of the 'inversion effects' of the system, but an investigation into this problem is proceeding and a partial solution will be reported later.

It may well be that the presence of those synergists, which have a mode of action similar to that of sesamin and isobutylundecylenamide, also influences, by the formation of complexes, the orientation of the pyrethrins molecules at the nerve-sheath interface, and it is hoped to follow this process by the changes in the surface (interfacial) potential which are expected to accompany it.

It has been reported by Dove (1947) that the pyrethrins are stabilized in thin films by certain synergists, particularly piperonyl butoxide. It may be that loose complex formation of the type suggested above, either at the oil-air or oil-substrate interfaces, reduces the photolytic tendencies of the pyrethrins molecules, since such a combination would tend to reduce the free energy of the insecticide molecules. It should, however, be borne in mind that the apparent toxicity of pyrethrum-oil films 'stabilized' by the addition of synergists such as sesamin and isobutylundecylenamide may be misleading if the threefold increase in the relative potency due to the presence of the synergist is not taken into account.

We are indebted to Dr A. Weed of John Powell Inc. for supplying the sesamin and I.N. 930 used.

REFERENCES

David, W. A. L. & Bracey, P. (1944). The activation of pyrethrins in fly sprays. *Nature, Lond.*, **153**, 594.

Dove, W. E. (1947). Piperonyl butoxide, a new and safe insecticide for the household and field. *Amer. J. trop. Med.* **27**, 339.

Haller, H. L., Laforge, F. B. & Sullivan, W. N. (1942*a*). Some compounds related to sesamin, their structure and their synergistic effect with pyrethrum insecticides. *J. org. Chem.* **7**, 185.

Haller, H. L., Laforge, F. B. & Sullivan, W. N. (1942*b*). Effect of sesamin and related compounds on the insecticidal action of pyrethrum on houseflies. *J. econ. Ent.* **35**, 247.

Haller, H. L., McGovran, E. R., Goodhue, L. D. & Sullivan, W. N. (1942). The synergistic action of sesamin with pyrethrum insecticides. *J. org. Chem.* **7**, 183.

HARTZELL, A. (1945). Histological effects of certain sprays and activators on the nerves and muscles of the housefly. *Contr. Boyce Thompson Inst.* **13**, 443.

HARTZELL, A. & SCUDDER, H. I., (1942). Histological effects of pyrethrum and an activator on the central nervous system of the housefly. *J. econ. Ent.* **35**, 428.

HARTZELL, A. & WEXLER, E. (1946). Histological effects of sesamin on the brain and muscles of the housefly. *Contr. Boyce Thompson Inst.* **14**, 123.

MUNRO, J. W. (1942). The entomology of commerce: insect pests and their control. *Analyst*, **67**, 155.

PAGE, A. B. P., STRINGER, A. & BLACKITH, R. E. (1946). Investigation of insecticidal sprays. *Nature, Lond.*, **161**, 81.

PAGE, A. B. P., STRINGER, A. & BLACKITH, R. E. (1949). Bioassay systems for the pyrethrins. I. Water-base sprays against *Aëdes aegypti* L. and other flying insects. *Ann. appl. Biol.* **36**, 225.

PARKIN, E. A. & GREEN, A. A. (1944). Activation of pyrethrins by sesame oil. *Nature, Lond.*, **154**, 16.

RICHARDS, A. G., Jr. (1941). Differentiation between the toxic and suffocating effects of petroleum oils on larvae of the household mosquito (*Culex pipiens*). *Trans. Amer. ent. Soc.* **67**, 161.

RICHARDS, A. G., Jr. (1943). Lipid nerve sheaths in insects and their probable relation to insecticidal action. *J. N.Y. ent. Soc.* **51**, 55.

(*Received* 30 *October* 1948)

THE FUNGISTATIC ACTIVITY OF ETHYLENIC AND ACETYLENIC COMPOUNDS

III. THE FUNGISTATIC ACTIVITY OF TETRAIODOETHYLENE AND RELATED COMPOUNDS

By IRENE MUIRHEAD

Imperial Chemical Industries Limited, Butterwick Research Laboratories, The Frythe, Welwyn, Herts

The high fungistatic activity of tetraiodoethylene has been investigated. Diiodoacetylene, which like tetraiodoethylene is an unsaturated substance containing only carbon and iodine, has also been found to have a high antifungal action. The effect of replacing the iodine atoms in these compounds by hydrogen, bromine, and carboxyl and nitro groups has been studied. The high fungistatic activity of some iodoethylenic and iodoacetylenic compounds appears to be associated with the presence in the molecule of positive iodine.

McGowan, Brian & Hemming (1948) studied the antifungal action of a considerable number of ethylenic and acetylenic compounds. They found that, in general, the presence of an electron-attracting substituent, such as a nitro group, attached to the unsaturated carbon induced high fungistatic activity. However, among the substances they examined was tetraiodoethylene which they found to be highly fungistatic, and since iodine does not possess a powerful electron-attracting characteristic they stated that 'it does not seem possible to attribute the high toxicity of this compound to the withdrawal of electrons from the double bond by the iodine atoms'. The high activity of tetraiodoethylene seems all the more surprising since tetrachloroethylene and tetrabromoethylene do not possess a correspondingly high activity against the test fungus.

The present paper reports the result of an investigation carried out in an attempt to explain the high biological activity of tetraiodoethylene.

Biological evaluation of various iodo-compounds

A variety of substances was examined including inorganic and non-ethylenic compounds containing iodine, an ethylenic substance containing iodine *not* attached to the unsaturated carbon atom and unsaturated substances containing iodine which is attached to the carbon atom(s) of the ethylenic or acetylenic linkage.

The method of biological evaluation described by McGowan *et al.* (1948) was used and the results are given in Table 1.

The compounds available in the laboratory were purified by the usual methods before testing for fungistatic activity. A number of the compounds were specially prepared, according to literature methods. The instructions of Biltz (1897) were

TABLE 1. *Fungistatic activity of various iodo-compounds*

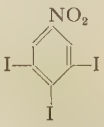
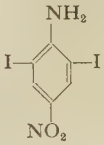
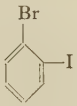
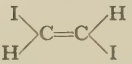
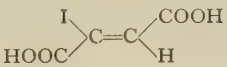
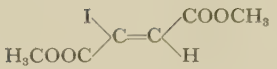
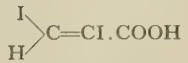
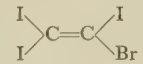
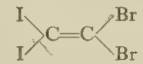
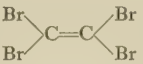
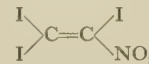
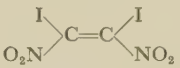
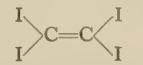
Code no.	Substance		*Botrytis allii* spore germination test. (Least conc. (μg./ml.) inhibiting growth)
1	Iodine	I_2	31·25
2	Potassium iodide	KI	> 1000
3	Iodic acid	HIO_3	> 1000
4	Carbon tetraiodide	CI_4	25
5	Iodoform	CHI_3	> 100
6	Isopropyl iodide		> 50
7	Trimethylene iodide	$I.CH_2CH_2CH_2I$	> 50
8	*n*-Nonyl iodide	$CH_3(CH_2)_8I$	> 100
9	Tetraiodopyrrole		> 100
10	3:4:5-Triiodonitrobenzene		> 100*
11	2:6-Diiodo-*p*-nitraniline		> 100
12	*o*-Bromoiodobenzene		Partial inhibition at 100
13	Iodobenzene		> 100
14	2:5-Diiodonitrobenzene		50
15	Allyl iodide	$CH_2{=}CH.CH_2I$	> 50
16	*sym.*-Diiodoethylene		> 250
17	Iodofumaric acid		> 100

TABLE 1 (*continued*)

Code no.	Substance		*Botrytis allii* spore germination test (Least conc. (μg./ml.) inhibiting growth)
18	Dimethyl iodofumarate		6·25
19	Triiodoacrylic acid		> 100
20	αβ-Diiodoacrylic acid		> 100
21	Triiodobromoethylene		25–50
22	*unsym.*-Diiododibromoethylene		50
23	Tetrabromoethylene		> 100
24	Nitrotriiodoethylene		0·19
25	*sym.*-Dinitrodiiodoethylene		6·5
26	Diiodoacetylene	I.C≡C.I	12
27	Iodopropiolic acid	I.C≡C.COOH	100
28	Methyl iodopropiolate	I.C≡C.$COOCH_3$	12·5
29	Tetraiodoethylene		12·5

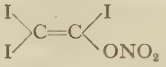
* Although 3:4:5-triiodonitrobenzene was not fungistatic at 100 μg./ml., stunting of germ-tubes of the test fungus was observed down to 6·25 μg./ml. concentration of the substance.

followed in the preparations of *sym.*-diiodoethylene, nitrotriiodoethylene, diiodoacetylene and tetraiodoethylene. For triiodoacrylic acid and αβ-diiodoacrylic acid the methods of Homolka & Stolz (1885) were used. Tetrabromoethylene, triiodobromoethylene and diiododibromoethylene were prepared as described by Nef (1897). The syntheses of iodofumaric acid and its methyl ester, dinitrodiiodoethylene and iodopropiolic acid were carried out by the methods of Thiele & Peter (1909), Biltz & Kedesdy (1900) and Nef (1899) respectively.

The methyl ester of iodopropiolic acid had not been previously prepared. Iodopropiolic acid (1 g.) was refluxed with methyl alcohol (2·85 ml.) and a few drops of concentrated sulphuric acid for 1 hr. The solution was then cooled and neutralized with sodium carbonate solution. The cream-coloured precipitate was

filtered off (0·74 g.), dried and recrystallized from petrol ether. (Found: C = 22·7%; H = 1·4%; I = 61·9%: $C_4H_3IO_2$ requires C = 22·9%; H = 1·4%; I = 60·5%.) The melting-point was 63° C.

In the same year as Biltz (1897) described the preparation of nitrotriiodoethylene, Nef announced the preparation of what he called triiodovinyl nitrate,



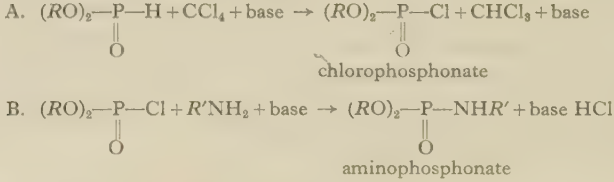
Later Biltz & Kedesdy (1900) brought forward evidence to show that the two compounds were identical and that the so-called triiodovinyl nitrate was actually nitrotriiodoethylene and contained less oxygen than Nef supposed. However, in a recent book Sartori (1939) refers to Nef's triiodovinylnitrate. Therefore, since there seemed to be still some doubt about the constitution, both methods of preparation were followed. The two products were of exactly the same colour and melting-point. Moreover, the melting-point of one was not depressed by admixture with the other, indicating that the two substances were identical. We are grateful to Mr J. Haslam of Imperial Chemical Industries Limited, Plastics Division, for undertaking an accurate iodine estimation of the compound. His figure of 84·50% iodine agrees well with 84·47% iodine, calculated for nitrotriiodoethylene. (Calculated for triiodovinyl nitrate, 81·58% iodine.)

Discussion

It will be seen from Table 1 that the mere presence of iodine in a compound does not necessarily induce high antifungal activity (Code nos. 1–14). Further, even compounds bearing a close resemblance to tetraiodoethylene (Code no. 29), and containing both iodine and a double bond are not all fungistatic (Code nos. 15–17 and 19). The replacement of iodine by hydrogen and/or carboxyl groups in tetraiodoethylene results in a great diminution of activity (Code nos. 16, 17, 19 and 20). The introduction of bromine in place of iodine also depresses the fungistatic activity but to a lesser extent than hydrogen (Code nos. 21–23). Substitution, however, does not always reduce antifungal action. Nitrotriiodoethylene (Code no. 24) has a much greater activity than tetraiodoethylene and so also has *sym.*-dinitrodiiodoethylene (Code no. 25), although its activity is less than that of nitrotriiodoethylene. This increased activity may be connected with the presence of the electron-attracting nitro group. Diiodoacetylene (Code no. 26) has an antifungal activity equal to that of tetraiodoethylene and like tetraiodoethylene contains only carbon and iodine as well as an unsaturated linkage. Substituting a carboxyl group for one of the iodine atoms in this compound gives iodopropiolic acid (Code no. 27) and the fungistatic activity is depressed just as in the ethylenic series. Dimethyl iodofumarate (Code no. 18) and methyl iodopropiolate (Code no. 28) are, however, highly fungistatic. Grove (1948) found that whereas bromofumaric acid and acetylene dicarboxylic acid had practically no fungistatic activity, the dimethyl and diethyl esters of these acids

were highly active. These are strong acids and, being largely ionized under the conditions of the test, are unable to penetrate to the site of action. A similar explanation would account for the above results. Carbon tetraiodide (Code no. 4), a third compound containing only carbon and iodine, was examined and found to have quite a high fungistatic activity. However, it is known that this compound decomposes very easily to give among other products, iodine, and this may account for the biological activity.

It is not quite certain what is the mode of fungistatic action of tetraiodoethylene and related compounds, but a very interesting suggestion was made to us by Prof. A. R. Todd. He suggested that some of these compounds might yield positive halogen, and that the biological action might be connected with this property. Atherton & Todd (1947) studied the phosphorylation of strong bases by diaryl or dialkyl phosphites in presence of a suitable halogen compound such as carbon tetrachloride. The reaction may be represented by the following equations:

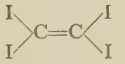
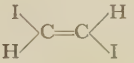
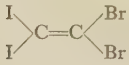
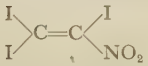
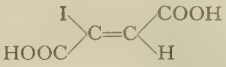


It seemed likely that in this reaction the carbon tetrachloride splits up into CCl_3 and Cl^+, and that the formation of the aminophosphonate depends on the ability of the halogen compound used in the reaction, to split off positive halogen. Mr V. M. Clark in Prof. Todd's laboratory at Cambridge tried this reaction with tetraiodoethylene and diiodoacetylene, using ammonia and dibenzyl hydrogen phosphite. Both these halogen compounds gave good yields of the dibenzylaminophosphonate, indicating that both these compounds can split off positive halogen. The biologically inactive tetrachloroethylene was found by Atherton & Todd (1947) to give no dibenzylaminophosphonate in the phosphorylation reaction.

Further work using the phosphorylation reaction was undertaken in this laboratory, and diisopropyl hydrogen phosphite (McCombie, Saunders & Stacey, 1945), which is easier to prepare and purify than dibenzyl hydrogen phosphite, was used. The substance under test and an equivalent amount of diisopropyl hydrogen phosphite were dissolved in a suitable solvent such as xylene, ether, or methylene chloride. Ammonia was used as the base and the dry gas was bubbled into the solution for 1 hr. If the reaction was exothermic the reaction mixture was cooled in water. The precipitated ammonium iodide was filtered off and the filtrate worked up for the diisopropylaminophosphonate. For example, in the case of tetraiodoethylene, the by-product is *sym.*-diiodoethylene which is very volatile in steam. The diisopropylaminophosphonate, which is not readily steam distilled, remained as an aqueous suspension. This was extracted with chloroform, the solution dried over sodium

sulphate and the chloroform distilled off under slightly reduced pressure. The residue crystallized as long colourless needles, m.p. 56–57° C. The material was then dried and weighed and identified by a mixed melting-point with an authentic sample. Sometimes the yields were not very good because the reaction was not complete in 1 hr. The results are listed in Table 2. From the table it will be seen that the highly active fungistats in this series can yield positive iodine. However, such positive iodine occurs in several compounds which have little or no fungistatic activity.

TABLE 2. *The phosphorylation reaction of various iodo-compounds*

Substance		Phosphorylation reaction	Fungistatic activity against *B. allii* (μg./ml.)
Tetraiodoethylene		+	12·5
sym.-Diiodoethylene		–	>250
Dibromodiiodoethylene		+	50
Nitrotriiodoethylene		+	0·19
Iodofumaric acid		–	>100
Allyl iodide	$CH_2{=}CH.CH_2I$	+ (slight)	>50
Iodoform	CHI_3	+	>100
Diiodoacetylene	$IC{\equiv}CI$	+	12
Iodopropiolic acid	$IC{\equiv}C.COOH$	+	100

The high fungistatic activity of tetraiodoethylene and related compounds does therefore appear to be connected with the presence in the molecule of positive iodine attached at the double bond. This activity can be depressed by certain substituents such as hydrogen, bromine and carboxyl groups, and greatly increased by the introduction of the electron-attracting nitro group. However, other factors must be concerned in the production of high fungistatic activity and these have still to be determined.

Three of these compounds have been examined for bacteriostatic activity and the results are given below in Table 3.

TABLE 3. *The bacteriostatic activity of various iodo-compounds*

	Least concentration (μg./ml.) inhibiting growth			
Substance	*Staphylococcus aureus*	*Salmonella typhi*	*Escherichia coli*	*Bacillus subtilis*
Tetraiodoethylene	3·125	—	12·5	—
Nitrotriiodoethylene	25	25	25	—
Diiodoacetylene	12·5	25	25	25

It should perhaps be mentioned that antiseptic properties have been attributed to tetraiodoethylene and carbon tetraiodide. It has been suggested by Brownlee (1927) that the latter compound has antiseptic and cleansing properties and might, with advantage, be incorporated in tooth pastes. Tetraiodoethylene has been termed diiodoform and has been described as an antiseptic in the Merck Index (1940) and the U.S. Dispensatory (1943). From these descriptions it was apparently believed that tetraiodoethylene resembled iodoform in its biological properties. However, it will be seen from Table 1 that whereas tetraiodoethylene (Code no. 29) is powerfully fungistatic, iodoform (Code no. 5) showed no activity in the *B. allii* test.

The author's thanks are due to Mr J. C. McGowan for much encouragement and advice, and to Dr P. W. Brian and Mr H. G. Hemming for the biological assays.

REFERENCES

ATHERTON, F. R. & TODD, A. R. (1947). Studies on phosphorylation. Part III. *J. Chem. Soc.* May, p. 674.

BILTZ, H. (1897). Dijodacetylen und Tetrajodäthylen. *Ber. dtsch. chem. Ges.* **30**, 1200.

BILTZ, H. & KEDESDY, E. (1900). Nitrotrijodäthylen und Dinitrodijodäthylen. *Ber. dtsch. chem. Ges.* **33**, 2194.

BROWNLEE, R. (1927). U.S. patents Numbers 1,645,791, 1,645,792 and 1,645,793.

GROVE, J. F. (1948). The fungistatic activity of ethylenic and acetylenic compounds. II. Esters of halogenofumaric acids and acetylene dicarboxylic acid. *Ann. appl. Biol.* **35**, 37.

HOMOLKA, B. & STOLZ, F. (1885). Ueber die Iodpropargylsäure. II. Substituirte Acrylsäure. *Ber. dtsch. chem. Ges.* **18** (Juli-Dec.), 2284.

MCCOMBIE, H., SAUNDERS, B. C. & STACEY, G. J. (1945). Esters containing phosphorus, Part 1. *J. chem. Soc.* June, p. 382.

MCGOWAN, J. C., BRIAN, P. W. & HEMMING, H. G. (1948). The fungistatic activity of ethylenic and acetylenic compounds. I. The effect of the affinity of the substituents for electrons upon the biological activity of ethylenic compounds. *Ann. appl. Biol.* **35**, 25.

NEF, J. U. (1897). Ueber das zweiwertige Kohlenstoffatom. Die Chemie des Methylens. C. Chemie des Acetylidens, $CH_2{=}C$. *Liebigs Ann.* **298**, 353.

NEF, J. U. (1899). Ueber das Phenylacetylen, seine Salze und seine Halogensubstitutionsproducte G. Versuche in der Acetylenreihe. *Liebigs Ann.* **308**, 325.

SARTORI, M. (1939). *The war gases, chemistry and analysis.* Translated by L. W. Marison. New York, 52.

THIELE, J. & PETER, W. (1909). Jodidchlorid und Jodosoverbindungen aus Chlorjodfumarsäure. *Liebigs Ann.* **369**, 122.

(*Received* 20 *October* 1948)

INVESTIGATION INTO THE PRODUCTION OF BACTERIOSTATIC SUBSTANCES BY FUNGI

A REVISION OF THE TESTING METHOD

By W. H. WILKINS

Mycology Laboratory, Department of Botany, University of Oxford

(With Plate 5 and 4 Text-figures)

Discrepancies in the comparative results of workers on antibacterial substances produced by fungi can usually be attributed to the use of different testing methods. The method described here for testing fungus metabolism solutions is designed to facilitate comparison in that its characteristic features are simplicity and rapidity. A nutrient broth of the standard ingredients of peptone, salt and water only, plus agar, ensures that a test involving 100 plates, i.e. 400 tests, can be set up by one assistant in 3 hr. The sensitivity of the test is almost equal to that of a dilution series and the results are consistent over a wide range of individual and circumstantial variation.

Since the above investigation was started early in 1941, the method of testing the reaction of bacteria to the antibacterial substances produced by fungi has been that already described (Wilkins & Harris, 1943). The method falls into the class generally referred to as an 'agar cup' or 'agar plate' method, the antibiotic being placed in a hole in bulk-seeded agar and its potency estimated by a measurable diffusion zone of bacterial inhibition. It has been referred to as 'a modification of the method used by Fleming', but as Fleming did not describe the method in any detail (Fleming, 1942), and as the present method was devised and in use before Fleming's paper appeared, it will be referred to here as the Wilkins method. The method was used originally in this laboratory for the testing of *Penicillia* and *Aspergilli*, but as the work has more recently been concerned with testing Basidiomycetes, it is now revised and restated as a method adapted for the testing of fungi in general and Basidiomycetes in particular. Since this method was first used, other somewhat similar methods have appeared, but one previously described by Abraham *et al.* (1941) and later more fully described by Heatley (1944) has been widely used for the assay of penicillin, and though not necessarily equally well suited to the testing of fungal products it has been so used, and in this paper will be referred to as a standard of comparison. The Heatley plates used in comparative experiments were kindly produced at the School of Pathology by Dr Heatley's assistants. The Heatley and the Wilkins tests differ in both media and method. The media, though basically similar, vary in the kind, amount and proportions of the ingredients, and the methods differ in that whereas Heatley puts the substance to be tested into a small cylinder on a plate of surface-seeded agar Wilkins puts the substance into a hole in bulk-seeded agar. In both

cases the test bacteria have usually been *Bacterium coli* and *Staphylococcus aureus* as in this paper. The past seven years of experimentation, during which several thousand tests have been made, have confirmed my opinion that not only is the bulk-seeded medium quicker to make up and simpler to manage than the surface-seeded medium, but the Hole Testing method is preferable to the cylinder method because, in the former, radial diffusion of the test liquid is more direct and therefore more rapid and the sensitivity of the test is thereby increased.

The discrepancies in results which are mentioned by all workers in this field appear to be mainly due to (*a*) the medium on which the fungus was grown or (*b*) the method of testing, or both. This paper deals only with (*b*). Testing metabolism solution of the same fungus culture (or subculture), or even testing the same metabolism solution by different methods rarely gave similar results. Hervey (1947), who did a survey of 500 Basidiomycetes, comments on the work of, among others, Mathieson (1946), Robbins *et al.* (1945), Wilkins (1945, 1946) and Wilkins & Harris (1944*c*) and says that her results do not agree with those of the workers mentioned. She says that to evaluate results it would be necessary to use subcultures of the particular isolations used by the other investigators.

After publication of papers on strain variation in *Aspergilli* by Wilkins & Harris (1944*d*) and by Furtado of Brazil (1945), which showed disagreement in that the former got positive results with all fourteen strains of *A. niger*, whereas the latter got negative results in all his thirty-two strains of that species, cultures were exchanged and retested. Furtado then got a negative result with twelve out of our fourteen 'positive' strains, while we got a positive result with twenty-two of his thirty-two 'negative' strains. Similarly, a set of seventy-seven *Penicillia* tested at the School of Pathology and in this Laboratory by the Heatley and the Wilkins methods respectively gave only 58% agreement. Of the 42% disagreement, 35% was positive with us, whereas it had been negative with them and 7% was the opposite. Again, Robbins *et al.* (1945) published an examination of 300 Basidiomycetes and kindly sent me sixty-six of these to test. When tested by the Robbins method, his results were confirmed, but when tested by the Wilkins method there was lack of agreement. The discussion has been published (Wilkins, 1947).

In the above three cases the same cultures (or subcultures) were used by both sides but quite different results were obtained. For complete evaluation of results it is desirable to use the same testing method.

Revision of media

This involved the composition and making up of nutrient broth and agar and the running of the bacterial suspension.

Nutrient broth and agar

Experimental evidence shows that a satisfactory nutrient medium could be made up from the ingredients used by Heatley and by Wilkins as other ingredients which

have been tried at various times showed no significant improvement. The two media are constituted as follows:

	Heatley	Wilkins
Lab. Lemco (g.)	10·0	5·0
Peptone (g.)	10·0	5·0
Sodium chloride (g.)	5·0	—
Dextrose (g.)	—	5·0
Phosphate buffer	5 % M/1·5	—
Indicator (%)	0·0025	—
Agar (g.)	20·0	20·0
Water (ml.)	Tap 1000·0	Dist. 1000·0

The effect of the quantity and proportion of each constituent was examined and the Wilkins-revised medium was based on the results as follows:

The use of oxoid peptone (Messrs Oxo Ltd.) made meat extract unnecessary but, perhaps due to elimination of the extract, sodium chloride was an improvement. Dextrose, never regarded as essential, was omitted largely because of the possibility of its decomposition by certain substances, e.g. notatin, with liberation of hydrogen peroxide and consequent inhibition of bacterial growth. Phosphate buffer solutions serve no useful purpose, but indicator, though not essential, is often useful. We find oxoid standard agar to be very satisfactory as it can be used in a 1·0% concentration. Though there was no apparent difference with tap or distilled water, the monthly analysis of Oxford tap water (courtesy H. H. Crawley, Esq., City Water Engineer) suggested the continuance of distilled water.

The Wilkins-revised medium

Oxoid peptone (g.)	20·0
Sodium chloride (A.R.) (g.)	5·0
Distilled water (ml.)	1000·0
Oxoid standard agar (g.)	10·0
Brom-thymol blue, stock solution (ml.)	50·0

(i) *The nutrient broth.* Dissolve the peptone and the sodium chloride in cold distilled water. If exceptionally clear broth is required filter through Whatman no. 1. Adjust to pH 7·2 by 2N sodium hydroxide. Put into suitable vessel, e.g. tube in 10 ml. quantities, plug and autoclave for 10 min. at 1 atmosphere. Store in cool place.

(ii) *Nutrient agar.* Add the agar after adjusting, and before sterilizing the broth. The stock solution of Brom-thymol blue is made by dissolving 0·1 g. dry stain in 250 ml. distilled water to which has been added 0·5 ml. of a 2N solution of sodium hydroxide.

The bacterial suspensions

(i) *Broth suspension.* Keeping the test bacteria in a state of uniform optimum activity with a relatively constant number of viables requires meticulous attention. The strains of *B. coli* and *S. aureus* are obtained from the Oxford School of Pathology. They are kept on slants of nutrient agar in the refrigerator and subcultured on to a fresh slant once a fortnight. The subcultures are kept for 24 hr. at 37°* before

* Unless otherwise stated, all temperatures are in ° C.

transference to refrigerator. They are examined microscopically at frequent intervals. Sterile broth from cold storage is put into the incubator so that it reaches a temperature of 37° before it is used for a suspension. Subculturing of the suspension is carried out every 24 hr. between 11.30 and noon. The time schedule used here is as below, *S* representing a slant, figures the tubes containing the suspension, and starred figures the suspension available for use:

Days	W.	Th.	F.	S.	Su.	M.	T.	W.	Th.	F.	S.	Su.	M.	T.	W.	Th.	F.	S.	Su.	M.
Subculture	S_1	1	2	3	4*	5*	6*	7*	8*	9*	10*	11*	12*	13*	14*	15*	16*	17*	18	19
															S_2	1	2	3	4*	5*

Needham (1947) stated that the broth suspension used for testing should have been through not less than four and not more than eighteen subcultures, and should have been incubated at 37° for not less than 23 and not more than 25 hr. I have arrived at the same general conclusion but am prepared to allow slightly more latitude.

(ii) *Agar suspension.* After sterilization, cool the agar to about 50°. Then put into a 45° incubator and allow to cool to this temperature. To each 100 ml. at 45° add 1 ml. of 24 hr. suspension of the required bacteria. Though a 24 hr. suspension is generally used, suspensions up to 72 hr. old can be used without affecting the results. Distribute the bacteria by gentle agitation and pour into plates immediately. In case of unavoidable delay, return to 45° incubator until required. It can be left in the incubator for up to 9 hr. without affecting the result.

The testing methods

We have certain aids to rapidity and precision of technique as follows:

(1) The Medium Distributing Apparatus which rapidly and accurately delivers 20 or 30 ml. into plates (Pl. 5, fig. 1).

(2) The Template (Text-fig. 1) which covers all stages of both tests. Bound in glass it lasts almost indefinitely and in practice has amply justified the small amount of trouble involved in making it.

(3) The Automatic Measuring Pourer (Text-fig. 2). This is fitted to top of flask, and delivers 20 ml. (or 30 ml. according to size) of bulk-seeded agar each time it is tipped up. Made by any good glass blower or from Messrs Gallenkamp, London.

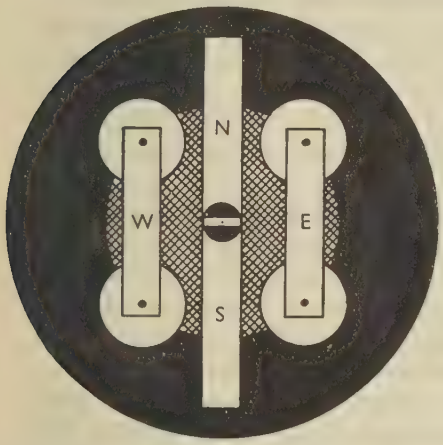
(4) The Viewing Box used for measuring results. The box is 9 in. square in plan and a sectional elevation is given in Text-fig. 3. The light chamber is painted with aluminium paint and the plate chamber is painted black.

The fungi are tested by (1) a preliminary Strip Test, and (2) the final Hole Test.

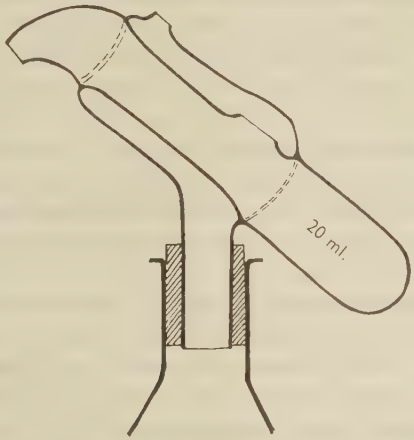
(1) *The Strip Test*

Originally devised by Wilkins & Harris (1944*b*) this has been considerably modified. It is regarded as a preliminary sorting test, and fungi which give a negative result are not tested further as they produce a negative or at best a feeble

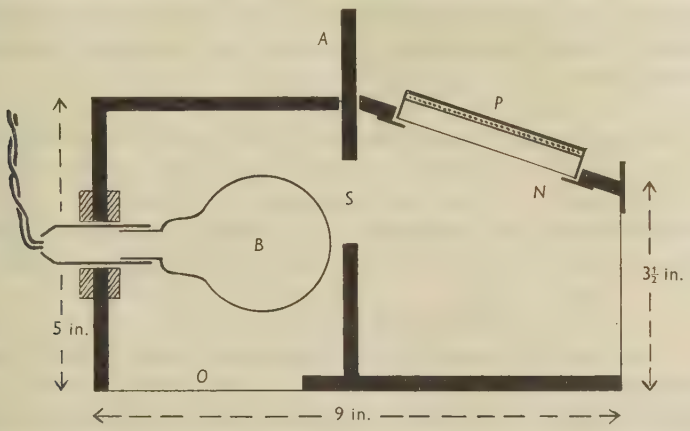
positive in any subsequent test. Robbins *et al.* (1945) use an essentially similar test but with a radially linear series of disks instead of a strip. They obtained significant results to which they attach considerable importance. Hervey (1947) also used this



Text-fig. 1. The Template. The black circle is the size of a standard Petri dish. Explanation in the text.



Text-fig. 2. The Automatic Measuring Pourer. Used for delivering measured quantities of bulk-seeded agar.



Text-fig. 3. The Viewing Box. Sectional elevation to scale. *A*, adjustable slide for regulating the width of the slit *S*; *B*, 40 W. pearl bulb; *O*, opening for changing bulb; *P*, Petri dish to be measured inverted over circular opening; *N*, small nails for supporting Petri dish.

method. We originally used a disk for preliminary testing (Wilkins & Harris, 1944*a*) but discarded it in favour of the strip.

The fungi for strip testing are grown in 30 ml. of malt agar (M) and potato dextrose agar (PD) until the colonies are about 2½ in. in diameter. Then 10 mm.

disks, cut equidistant from the growing edge, are placed in paired sterile plates, one disk per plate, surrounded with 30 ml. M and 30 ml. PD respectively and incubated at 20 or 25° until the colony has reached a standard size as indicated by the cross-hatched circle in the centre of the template. They are then ready for testing.

Using the template, with the strip-cutting apparatus of two parallel scalpels, an 8 mm. strip is cut across the diameter of the colony in each of a pair of plates containing M and PD (Pl. 5, fig. 2*a*). The strips are cut transversely at the centre and at each end into two equal half-strips *N* and *S* each 40 × 8 mm. The *N* half-strips are then transferred to the *W* position, as indicated by the template, in a pair of sterile plates and the *S* half-strips to the *E* position in the same pair of plates (Pl. 5, fig. 2*b*). Repeat with all the fungi to be tested. By means of the Automatic Measuring Pourer the strips are surrounded by 20 ml. of bulk-seeded agar so that in each pair of plates, one plate will have a half-strip *N* from medium M and a half-strip *N* from PD, in agar bulk-seeded with *B. coli* and the other plate will have corresponding half-strips *S* and *S* in agar bulk-seeded with *S. aureus*.

The plates are incubated in a single layer on asbestos-sheet shelves at 37° overnight. The degree of antibacterial activity is expressed by measuring with a glass scale across the widest part of the area of inhibition to the nearest 0·5 mm. The widest part varies with strips of different fungi, from different media, and against different bacteria. A typical strip test result is shown in Pl. 5, fig. 2*c*. While it is easy to see the widest part of the area of inhibition in any given instance (cf. Pontecorvo, 1945) identical results are not invariable even under what appear to be identical circumstances. I am reluctant to regard this test as an adequate and final indication of antibacterial activity.

(2) *The Hole Test*

This is used for testing the metabolism solutions produced by those fungi that have survived the Strip Test and is regarded as the ultimate criterion as to whether or not a fungus produces antibacterial substances which are likely to merit subsequent chemical isolation and purification. It has been in use since it was devised in 1941 (Wilkins & Harris, 1943), but the present revision will supersede the original.

Growing the fungi for the Hole Test

Disks, 10 mm. diameter, are cut just inside the growing margin of the colony used for strip testing. The disks are grown in 25 ml. of liquid media M and PD in 50 ml. conical flasks. The size of the flask, the quantity of medium and the number of disks per flask all have some influence on the result. This is being investigated in detail for publication later. For the present it can be said that, in the case of a positive fungus, one disk per vessel will produce its peak production of antibacterial substance on any quantity of medium from 10 to 1000 ml. in approximately the same time, which is usually between 25 and 35 days according to the fungus. This compares with the work of Whinfield (1948) who, using a dense inoculum of conidia (C), and also inocula diluted C/10, C/100 and C/1000, found that maximum penicillin

production was attained simultaneously in all cultures. For *Aspergilli* and *Penicillia* which grow quickly, it was customary to use 10 ml. of medium in a 50 ml. flask. For slow-growing fungi like Basidiomycetes, 10 ml. is not enough as the medium dries up before the fungus is fully grown, particularly as each test removes some of the metabolism solution. We find that one disk on 20 or 25 ml. in a 50 ml. flask is economical in time and space, and is the most satisfactory for routine testing.

The revised Hole Test

Take twice the number of plates as fungi to be tested. Using the Automatic Measuring Pourers, pour half the plates with 20 ml. of nutrient agar bulk-seeded with *B. coli*, and the other half with nutrient agar seeded with *S. aureus*. When the agar has gelled, using the template, cut four holes with a sterile 10 mm. cork borer in each plate at the points indicated on the template by the centres of the four white circles. With a sterile pipette (we use quill tubing drawn out and bent to a convenient angle), take a small amount of metabolism solution from each of the two flasks containing (say) fungus A on medium M and fill the two upper holes in the *B. coli* plate and the two upper holes in the *S. aureus* plate, one hole from each flask on each plate. Similarly, take metabolism solution from the flask containing the same fungus on medium PD and put into the two lower holes in each plate. There are now duplicate holes in each plate containing metabolism solutions of the given fungus from duplicate flasks of medium M and PD. Repeat the process with the other metabolism solutions until all the plates have been filled. Incubate in a single layer on asbestos-sheet shelves at 37° overnight. Measure on Viewing Box. Results are usually expressed as the total diameter of the area of inhibition including the hole. Zone width is obtained by subtracting the diameter of the hole and dividing by two. In this paper only zone widths are used to express results. A typical result of this test is shown in Pl. 5, fig. 3.

Precautions

In the above test certain processes allow some latitude while others admit of none. Some of these have been mentioned in the relevant text. Vesterdal (1946) has given a list of the precautions to be taken in this type of test if accurate results are to be expected. I am in agreement with some but not all of the points he mentions. In the Wilkins method the following merit special attention:

(1) The most important factor is the adjustment of the reaction of the agar suspension used for the test plates. Table 1 shows the effect on zone width produced by mercuric chloride, 1/6400, lactic acid (A.R.), 1/32, penicillin, 1/1 and metabolism solution of *Coprinus quadrifidus*, CBS 52, on Wilkins-R medium adjusted to three different initial pH values. The penicillin used is the pure calcium salt kindly supplied by the Oxford School of Pathology. The stock solution of 0·003 g. salt in 300 ml. sterile distilled water is kept in refrigerator. The 1/1 solution used for testing is made up by adding 1 ml. of stock solution to 15 ml. distilled water and contains 1 Oxford Unit per ml. The figures are the average of five plates.

The variation of the pH of the medium has a marked effect on the zone width. A variation of the medium towards alkalinity has less effect than an equal variation towards acidity. In the case of the test of metabolism solution CBS 52, a variation of pH 0·4 on the acid side, or pH 0·6 on the alkaline side of the normal pH 7·2, produces a difference of 1 mm. in zone width.

(2) For comparable results the size of the hole must be constant. Any sized borer between 8 and 18 mm. can be used, but we have found 10 mm. to be most convenient. It cuts easily and does not take much metabolism solution to fill it. Formerly, four drops were put into each hole but as the size of the drop varied with the size and method of holding the pipette, this has been discontinued. The holes are filled almost to the top, and as long as the meniscus reaches to the top, minor variations in the amount of liquid make no difference to zone width. Filling the hole to the top induces danger of spilling.

TABLE 1. *Effect of* pH *on zone width*

pH of agar suspension ...		6·2	7·2	8·2
		Zone widths in mm.		
Test liquid	pH			
Mercuric chloride	6·2	5·5	4·5	4·0
Lactic acid	1·8	9·5	7·0	5·5
Penicillin	6·6	6·0	5·0	5·0
CBS 52	6·8	10·5	7·0	5·5

TABLE 2. *Effect of standing time on zone width*

(Zone width in mm., average of 8 plates.)

ST (min.)	Mercuric chloride	Lactic acid	Penicillin
0	4·7	8·7	5·3
15	4·7	8·8	5·3
30	4·7	8·8	5·3
60	4·7	8·7	5·5

(3) Brownlee *et al.* (1948) stated that 'the time of standing before and after filling the plates' affected the result. Our plates are filled on the asbestos-sheet shelves and each shelf is put into the incubator as soon as finished. The time for filling one shelf of plates is approximately 10 min. and there is no appreciable difference in results due to standing time. This question of 'standing time after filling and before incubating' (*ST*) has been further checked against three test liquids (dilution as before) at different times at laboratory temperature as shown in Table 2.

It will be seen that even after an hour's standing there is no significant difference in results. Plates can stand for up to 4 hr. before filling without effect on the results.

The consistency of the Wilkins-revised test

By consistency is meant a high degree of probability that the Hole-Testing method, when carried out exactly as stated, will give consistent results though done by different people in different places and at different times. Three research assistants, *A*, *B* and *C* tested for consistency, on a limited scale, by carrying out a complete test of thirty plates with each of three test liquids (dilution as before) against *S. aureus*. The test liquids were common to all, otherwise the individual tests were done independently on different dates and the results were kept secret until the three tests were completed. The experiment was repeated making a total of 180 plates with each test liquid. The results represent the diameter of the total area of inhibition and are expressed as the mean and the standard deviation by the formula $\sigma = \sqrt{[\Sigma (v^2)/n - 1]}$ of each set of thirty observations. They are summarized in Table 3, where column I gives the results of each set of observations, column II the result of the two sets of each individual worker, and column III the result of all the observations with each test liquid.

TABLE 3. *Consistency test*

			I	I	III
Mercuric chloride	*A*	1	20·13 ± 0·39	19·67 ± 0·60	
		2	19·23 ± 0·39		
	B	1	18·87 ± 0·35	19·18 ± 0·49	19·53 ± 0·60
		2	19·48 ± 0·40		
	C	1	20·17 ± 0·38	19·72 ± 0·56	
		2	19·27 ± 0·29		
Lactic acid	*A*	1	23·22 ± 0·32	23·24 ± 0·33	
		2	23·27 ± 0·31		
	B	1	21·97 ± 0·22	22·56 ± 0·66	23·02 ± 0·58
		2	23·15 ± 0·34		
	C	1	23·25 ± 0·43	23·27 ± 0·38	
		2	23·28 ± 0·34		
Penicillin	*A*	1	24·57 ± 0·39	24·75 ± 0·36	
		2	24·93 ± 0·24		
	B	1	24·20 ± 0·45	24·27 ± 0·41	24·42 ± 0·49
		2	24·35 ± 0·37		
	C	1	23·92 ± 0·42	24·23 ± 0·50	
		2	24·53 ± 0·37		

The constancy as illustrated above is such as to need no further comment.

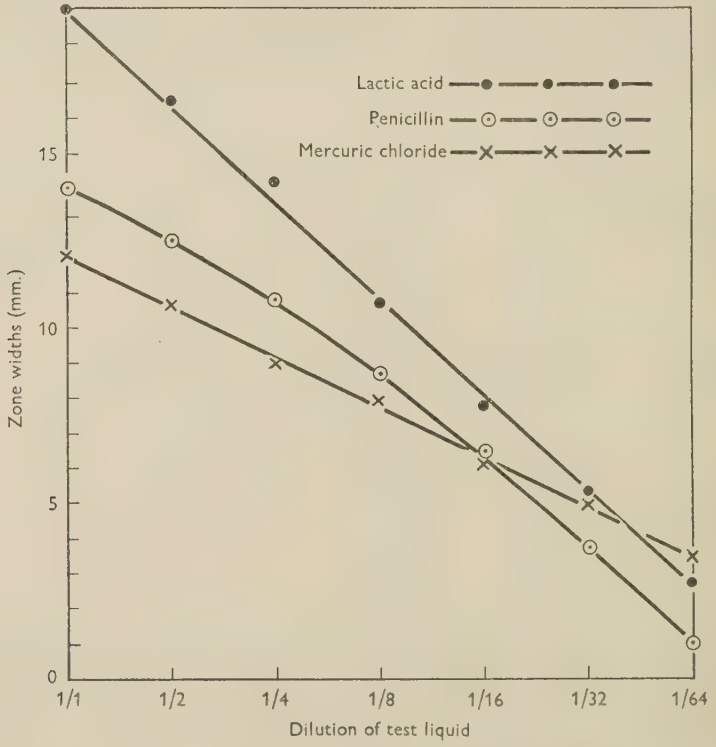
Personal variation in measuring test plates

The three assistants, *A*, *B* and *C*, each carried out a hole test of thirty plates against mercuric chloride and lactic acid as before, and measured the diameter of the area of bacterial inhibition. Each set was then measured by the other two assistants. This gave a total of 270 observations with each test liquid. The degree of

agreement was checked for each assistant's set of thirty plates by taking the agreement between $A:B$, $A:C$ and $B:C$ respectively. The results are summarized in Table 4.

TABLE 4. *Personal variation in measuring test plates*

	A's plates			*B*'s plates			*C*'s plates			
	A:B	*A:C*	*B:C*	*A:B*	*A:C*	*B:C*	*A:B*	*A:C*	*B:C*	Total
				Mercuric chloride						
Agreement	25	19	24	14	14	12	20	12	18	158
Disagreement	5	11	6	16	16	18	10	18	12	112
				Lactic acid						
Agreement	18	12	23	23	24	21	22	13	17	173
Disagreement	12	18	7	7	6	9	8	17	13	97



Text-fig. 4. Dilution graphs of the test liquids.

Theoretically there should have been agreement in the 270 observations. Actually there was often disagreement to the extent of 0·5 mm., and in a very few instances to 1·0 mm. This lack of agreement was attributable to the personal error in estimating the 0·5 mm., on the scale. Certain assistants attempted to measure to 0·5 mm., while

others consistently approximated to the nearest whole number. Some sets of plates appeared to be easier to measure than others, e.g. in *A*'s plates with mercuric chloride, the agreement is 87%, while in *B*'s plates in the same series there is a 56% disagreement. The test liquid also affected the result. Lactic acid, which gives a sharp delineation of the inhibition area, gave 64% agreement, whereas mercuric chloride with a less clear demarcation, gave only 58% agreement. Taking an example of disagreement such as *A*:*C* on *C*'s plate, the standard deviation, worked out as before is ±0·36, whereas the actual experimental average deviation is ±0·35. It seems, therefore, that apparent lack of agreement is largely due to error of individual measurement.

The high degree of consistency given by the test liquids makes it possible to use these for standardizing any given test with an unknown solution. The graphs resulting from plotting plate dilution tests for each liquid are illustrated in Text-fig. 4.

Comparison between the Heatley and Wilkins methods

This comparison was between (*a*) Wilkins-Original and Heatley and (*b*) Wilkins-Revised and Heatley, incidentally involving a comparison between the original and the revised Wilkins methods. In each case three test liquids were tested in duplicate against *S. aureus* only, on both the Heatley and Wilkins media and by the Heatley and Wilkins method in each case. In (*a*) the test liquids were mercuric chloride 1/200, penicillin 1/1 and a strongly positive metabolism solution of *Fomes annosus*. In (*b*) the first two were as in (*a*) but the SP metabolism solution was *Coprinus quadrifidus*. One duplicate was tested in the normal way, the other was refrigerated for 16 hr. after adding the test liquid but before incubating. Refrigeration increased the zone width particularly on the Heatley medium, but the information derived from the refrigeration comparison was not significant enough to warrant record. As a matter of interest, cylinders on bulk-seeded and holes in surface-seeded media were tried. Neither of these arrangements showed any advantage over the methods in common use and the results are not recorded. The comparative experiments (*a* and *b*) involved about 1000 plates, but the results have been summarized in Table 5 where each figure represents zone width and is an average of at least ten plates.

Table 5. *Comparison between the Heatley and the Wilkins testing methods*

		Mercuric chloride		Penicillin		Metabolism solution	
		Heatley	Wilkins	Heatley	Wilkins	Heatley	Wilkins
		(*a*) Heatley v. Wilkins-Original method					
Medium	Heatley	7·5	9·0	7·5	6·0	2·0	2·0
	Wilkins	8·5	8·5	6·0	6·0	7·0	6·5
		(*b*) Heatley v. Wilkins-Revised					
Medium	Heatley	12·5	12·5	7·0	6·0	5·0	6·0
	Wilkins	8·5	9·5	6·0	6·5	5·5	7·5

In the above table, any discrepancy seemed to be a function of the medium rather than of the method and, though direct correlation has not been established, the general degree of agreement between the two methods is satisfactory.

A dilution series of sixteen tubes was set up with several test liquids serially diluted by 1/2 with nutrient broth. From each tube a plate test was made by the Heatley and the Wilkins methods respectively. The tubes were also inoculated with a loop of given bacterial suspension and antibacterial activity was estimated by turbidity after an incubation of 24 hr. Table 6 gives the result for one test liquid as far as tube no. 12.

TABLE 6. *Comparative dilution/zonation test, Heatley and Wilkins-Revised*

Mercuric chloride 1 g. in 200 ml. water

Tube	1	2	3	4	5	6	7	8	9	10	11	12
					(*a*) *B. coli*							
Dilution	—	—	—	—	—	—	—	—	—	—	++	++
Zonation: Heatley	4·0	3·25	2·0	1·0	—	—	—	—	—	—	—	—
Wilkins	9·5	7·5	6·0	5·0	4·25	3·5	3·0	2·0	1·0	—	—	—
					(*b*) *S. aureus*							
Dilution	—	—	—	—	—	—	—	—	—	—	—	++
Zonation: Heatley	7·5	6·5	6·0	5·0	3·5	2·5	0·5	—	—	—	—	—
Wilkins	11·5	9·5	8·5	7·5	6·5	5·0	3·5	2·5	1·5	0·5	—	—

The Wilkins method shows a considerable degree of sensitivity in the zonation series. The agreement between dilution and zonation is very close, there being only one gap between the end of zonation and the beginning of turbidity. The Heatley method does not show up well against the mercuric chloride but against penicillin (not shown) the Heatley series is as sensitive as the Wilkins.

I am very grateful to the Medical Research Council for a grant enabling me to employ assistants for this work, and I am most appreciative of the valuable help given by the graduate assistants, Miss B. M. Partridge and Mr G. A. Salt, and by the technical assistants Miss J. F. Rose and Miss H. A. Tobin.

REFERENCES

ABRAHAM, E. P., CHAIN, E., FLETCHER, C. M., GARDNER, A. D., HEATLEY, N. G., JENNINGS, M. A. & FLOREY, H. W. (1941). Further observations on penicillin. *Lancet*, **2**, 117.

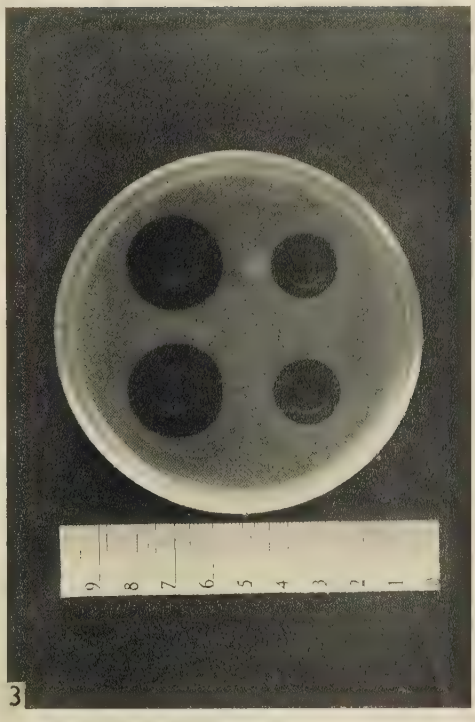
BROWNLEE, K. A., DELVES, C. S., DORMAN, M., GREEN, C. A., GRENFELL, E., JOHNSON, J. D. A. & SMITH, N. (1948). The biological assay of streptomycin by a modified cylinder plate method. *J. gen. Microbiol.* **2**, 40.

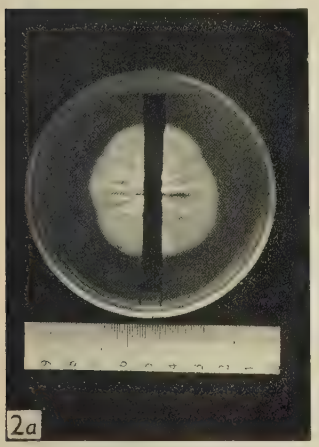
FLEMING, A. (1942). *In vitro* tests of penicillin potency. *Lancet*, **1**, 732.

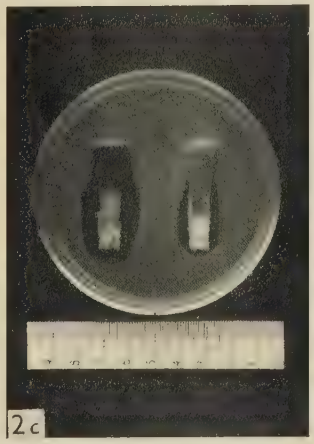
FURTADO, A. DA ROCHA (1945). Pesquisa da atividade antibacteriana com 180 amostras de *Aspergillus* Micheli 1729. *Mem. Inst. Osw. Cruz.* **41**, 205.

HEATLEY, N. G. (1944). A method for the assay of penicillin. *Biochem. J.* **38**, 61.

HERVEY, ANNETTE H. (1947). A survey of 500 Basidiomycetes for antibacterial activity. *Bull. Torrey bot. Cl.* **74**, 476.







WILKINS—*Production of bacteriostatic substances by fungi*

MATHIESON, JEAN (1946). Antibiotics from Victorian Basidiomycetes. *Aust. J. exp. Biol. med. Sci.* **24**, 57.

NEEDHAM, N. V. (1947). A photometric method for the comparative evaluation of disinfectants. *J. Hyg., Camb.*, **45**, 1.

PONTECORVO, G. (1945). Assay of the rates of secretion of antibiotics in different regions of a growing mould colony. *Nature, Lond.*, **155**, 515.

ROBBINS, W. J., HERVEY, A., DAVIDSON, R .W., MA, R. & ROBBINS, W. C. (1945). A survey of some wood-destroying and other fungi for antibacterial activity. *Bull. Torrey bot. Cl.* **72**, 165.

VESTERDAL, J. (1946). The agar cup method for the estimation of penicillin. *Acta Pharmacol. et Toxicol.* **2**, 9.

WHINFIELD, BARBARA (1948). Studies in the physiology and morphology of *Penicillium notatum*. *Ann. Bot., Lond.*, N.S. **12**, 111.

WILKINS, W. H. (1945). Investigation into the production of bacteriostatic substances by fungi. Cultural work on Basidiomycetes. *Trans. Brit. mycol. Soc.* **28**, 110.

WILKINS, W. H. (1946). Investigations into the production of bacteriostatic substances by fungi. Preliminary examination of the fifth 100 species, all Basidiomycetes, mostly of the wood-destroying type. *Brit. J. exp. Path.* **27**, 140.

WILKINS, W. H. (1947). Investigation into the production of bacteriostatic substances by fungi. Preliminary examination of the seventh 100 species, all Basidiomycetes. *Brit. J. exp. Path.* **28**, 247.

WILKINS, W. H. & HARRIS, G. C. M. (1943). Investigations into the production of bacteriostatic substances by fungi. A method for estimating the potency and specificity of the substances produced. *Ann. appl. Biol.* **30**, 226.

WILKINS, W. H. & HARRIS, G. C. M. (1944*a*). Estimation of the antibacterial activity of fungi that are difficult to grow on liquid media. *Nature, Lond.*, **153**, 590.

WILKINS, W. H. & HARRIS, G. C. M. (1944*b*). A modification of the method for estimating the anti-bacterial activity of fungi that are difficult to grow on liquid media. *Nature, Lond.*, **154**, 578.

WILKINS, W. H. & HARRIS, G. C. M. (1944*c*). Investigation into the production of bacteriostatic substances by fungi. Examination of the larger Basidiomycetes. *Ann. appl. Biol.* **31**, 261.

WILKINS, W. H. & HARRIS, G. C. M. (1944*d*). Investigation into the production of bacteriostatic substances by fungi. Preliminary examination of the third 100 fungi with special reference to strain variation among species of *Aspergillus*. *Trans. Brit. mycol. Soc.* **27**, 113.

EXPLANATION OF PLATE 5

Fig. 1. The Medium Distributing Apparatus. Hot agar from the spherical vessel is run into the graduated tube from which it is delivered into plates in 20 or 30 ml. quantities.

Fig. 2. The Strip Test Method: (*a*) strip cut across a colony on malt agar; (*b*) two half-strips, one from M (left) and one from PD (right) put into plate and surrounded by agar bulk-seeded with *S. aureus*; (*c*) the post-incubation result of the test showing a greater reaction against *S. aureus* on the strip from M (left) than on the strip from PD (right).

Fig. 3. Hole Test Result. Duplicate tests showing reaction of metabolism solution from fungus in M (left) and from the same fungus in PD (right), both against *S. aureus*.

(*Received* 23 *October* 1948)

THE TRANSMISSION OF SUGAR-BEET YELLOWS VIRUS BY MECHANICAL INOCULATION

By B. KASSANIS

Rothamsted Experimental Station, Harpenden, Herts

Sugar-beet yellows virus, hitherto transmitted only by aphides, can be transmitted mechanically to sugar-beet if a suitable abrasive is used. Most inoculated leaves develop local lesions, but systemic infection usually occurs in only 10% of the inoculated plants. Systemic symptoms develop more slowly than in plants infected with aphides.

Many workers have reported transmission of sugar-beet yellows virus by aphides, but there is no record of mechanical transmission, and several statements of failure to induce infection by inoculating healthy plants with sap from diseased sugar beet (e.g. Quanjer, 1934, 1936; Roland, 1939). The virus is one that persists in the aphis vector (Watson, 1940), and Smith & Lea (1946) have suggested that mechanical transmission fails with most of the persistent viruses because they occur in infected plants at too low a concentration. From the specific serological reactions obtained with sap from sugar-beet plants infected with yellows, however, it seems unlikely that this explanation adequately accounts for the behaviour of beet yellows virus. Kleczkowski & Watson (1944) have recorded precipitin titres varying between 1/16 and 1/64, values higher than those obtained with some other viruses that are readily transmitted mechanically. Thus if the specific antigen is the virus, which seems probable, some explanation other than a low virus content is necessary.

Attempts to infect sugar-beet plants mechanically were made by various methods. The inoculum was freshly expressed sap from yellowed sugar-beet leaves and the test plants were young seedlings of the variety Kleinwanzleben E, used when they had 4–5 leaves about 6–10 cm. long. As found by previous workers, plants showed no symptoms when the inoculum was simply rubbed over leaves of healthy beet, but when the leaves were dusted with celite or 400-mesh carborundum before being rubbed they became infected. The plants reacted differently from those infected by means of aphides, and most often symptoms were confined to the rubbed leaves.

Symptoms develop slowly, local lesions first appearing in not less than 2 weeks. The lesions take various forms, and the numbers produced on apparently similar plants used in one test may vary widely. Some leaves may show no clearly defined lesions, whereas others may show more than 100. Usually the lesions are small brown necrotic spots, but sometimes they are purplish, similar to those described by Watson (1940) as occurring occasionally where an infective aphid has fed. The infected leaves turn yellow and become brittle; sometimes this is the only symptom and occurs without any previous spotting. Sap from yellowed leaves precipitates

specifically with antiserum to sugar-beet yellows virus, sometimes giving a titre of 1/16.

Most of the inoculated plants show symptoms only on the rubbed leaves, the new growth appearing normal and being apparently virus-free, but usually about 10% become systemically infected. Plants infected by aphides show systemic symptoms after 8–12 days, but those infected by rubbing with an abrasive show symptoms on the young leaves only after 4–6 weeks. These symptoms are similar to those in plants infected by means of aphides, namely a vein clearing succeeded by chlorosis. Sap from these leaves gives the usual positive precipitin reaction with virus antiserum.

Bawden & Roberts (1948) have shown that the susceptibility of plants to infection with some viruses is increased by keeping them in the dark before inoculation. When sugar-beet plants were kept for 4 days in the dark before inoculation, three times as many became systemically infected with yellows virus as when plants were kept under normal glasshouse conditions. The plants kept in the dark did not show any necrotic local lesions; this may explain why no symptoms were seen in plants inoculated during the winter.

Hoggan's (1933) results when transmitting cucumber mosaic virus to sugar-beet in some ways resemble those obtained with yellows virus, for she found that transmission by aphides regularly produced systemic infection whereas mechanical inoculation gave only a few local lesions. Various other workers have also found it difficult to transmit cucumber mosaic virus from infected beet to other plants by sap inoculation. These results cannot be interpreted with any certainty, but it seems that two different factors may be interfering with the infection of sugar-beet by yellows and cucumber mosaic virus, one hindering the initial establishment of the viruses in the rubbed leaves and the other affecting the movement of particles out of these leaves. The first may well be explained by the presence in sugar-beet sap of substances that act as inhibitors of infectivity (Kassanis & Kleczkowski, 1948), for these inhibitors may be enough to prevent infection when sap is rubbed over leaves but not when the susceptibility of the rubbed leaves is greatly increased by the use of suitable abrasives. The usual localization of infection in plants inoculated with the help of abrasives suggests that virus either passes with difficulty from parenchymatous cells into the phloem or that movement along the phloem is in some way restricted. Quanjer (1934) has described a 'gummosis' of the sieve tubes in plants suffering from yellows and this may play a part in restricting movement. The ready occurrence of systemic infections when transmission is effected by aphides may be because the aphis during feeding places the virus directly into the phloem, before this tissue has become affected by the disease.

REFERENCES

BAWDEN, F. C. & ROBERTS, F. M. (1948). Photosynthesis and predisposition of plants to infection with certain viruses. *Ann. appl. Biol.* **35**, 418.

HOGGAN, I. A. (1933). Some viruses affecting spinach and certain aspects of insect transmission. *Phytopathology*, **23**, 446.

KASSANIS, B. & KLECZKOWSKI, A. (1948). The isolation and some properties of a virus-inhibiting protein from *Phytolacca esculenta*. *J. gen. Microbiol.* **2**, 143.

KLECZKOWSKI, A. & WATSON, M. A. (1944). Serological studies on sugar-beet yellows virus. *Ann. appl. Biol.* **31**, 116.

QUANJER, H. M. (1934). Enkele kenmerken der 'vergelings' ziekte van usiker-en voederbieten ter onderscheiding van de 'zwarte hontraten'-ziekte. *Tijdschr. PlZiekt.* **40**, 40.

QUANJER, H. M. (1936). Geschiedenis van het onderzoek over de vergelingsziekte en de mozaiekziekte van de biet. *Tijdschr. PlZiekt.* **42**, 45.

ROLAND, G. (1939). Recherches effectuées en 1937 sur la jaunisse et quelques carences minérales de la betterave et de l'épinard. *Tijdschr. PlZiekt.* **45**, 1.

SMITH, K. M. & LEA, D. E. (1946). The transmission of plant viruses by aphides. *Parasitology*, **37**, 25.

WATSON, M. A. (1940). Studies on the transmission of sugar-beet yellows virus by the aphis, *Myzus persicae* (Sulz.). *Proc. Roy. Soc.* B, **128**, 535.

(*Received* 15 *October* 1948)

THE COMPETITION BETWEEN BARLEY AND CERTAIN WEEDS UNDER CONTROLLED CONDITIONS

III. COMPETITION WITH *AGROSTIS GIGANTEA*

By HAROLD H. MANN and T. W. BARNES

Woburn Experimental Station, Husborne Crawley, Bletchley

The twitch grass *Agrostis gigantea* var. *dispar* occurs commonly as a serious weed on the light semi-acid soils found on the lower greensand near Woburn, Beds. It spreads rapidly by means of rhizomes, rarely forming stolons. All the rhizome growth takes place within 6 in. of the surface: 1 g. dry weight of roots and rhizomes may occupy up to 260 c.c. of soil, *A. gigantea* is slightly more easily eradicated than *Holcus mollis*.

When barley and this *Agrostis* are planted together, with abundance of water and nutrients for both, it is found that increasing the density of planting of the barley reduces the crop loss due to *Agrostis* competition, but the weed can, nevertheless, diminish the yield of close-planted barley by about a quarter. In a sparse crop of barley, any increase in *Agrostis* growth is reflected in an almost equal reduction in yield of barley. In proportion to its relative density the barley secures a larger share of the soil nitrogen than does the *Agrostis*.

Evidence is given suggesting that there is a marked effect of the one grass on the other even when there is no competition for root space and an ample supply of water and nutrients.

Introduction

There are two grasses with underground rhizomes (twitch grasses) growing as persistent weeds in the light semi-acid land on which the Woburn Experimental Station is situated. These are *Holcus mollis*, the subject of a previous paper in the present series (1947), and an *Agrostis* which we called *Agrostis stolonifera* in the paper cited. A more exact determination recently made at Kew has identified this grass as *A. gigantea* Roth. var. *dispar* (Michx.) Philipson, according to the latest monograph of this genus by Philipson (1937). The present paper deals with the competition of barley with this species.

The importance of the *Agrostis* named, as a weed among corn crops, has long been known, at least in the region of the lower greensand soils. Under the name of creeping bent grass, white bent, or black couch grass, it was described by Sinclair (1824) who noted that the root was 'strongly creeping' and that 'simple ploughing was ineffectual' in rooting out the weed in clayey soils. He could only recommend that men should follow the plough and fork out the roots. He recognized that it was a productive grass, as he found at the time of flowering it would yield nearly 3 tons of grass per acre. Even earlier than Sinclair, Stokes had noticed that this is the 'couch grass of ploughed fields which gives the farmer so much trouble to extirpate' (see

Philipson, 1937, note), and Brenchley (1920) notes that species of *Agrostis* are equally distributed on all types of soil, that *A. stolonifera* is quite often dominant on peat soils, and that it is more prevalent among wheat than among barley.

So far as our experience goes, it was found to appear and persist on the permanent wheat and barley plots at Woburn many years ago. It was at first overshadowed among the wheat plots by *Holcus mollis*, which grew more strongly, especially on the more acid of the plots in question. But, as the years went on, even on the more acid soil whose pH value had gone down to about 4·2, the proportion of this *Agrostis* gradually increased until it became almost the dominant twitch in the area. In 1945 a great effort was made to dig out both of these twitches, but in the following year *Holcus mollis* again dominated the situation. Digging out is, in fact, much more effective as a means of clearing this grass than it is with *H. mollis*. *Agrostis gigantea* is, however, a very serious twitch which has abundant strong rhizomes (though these are not so stout as those of *Holcus mollis*) which permeate the ground to a depth of about 6 in., while the fibrous roots penetrate very much farther. The rhizomes spread radially from the crown of the plants and have well-defined scale leaves. They are only occasionally branched and stolons are very infrequent. The grass has been specially prominent at Woburn both on the ordinary farm land with a pH value of about 5·8 and also on the more acid plots where the pH is below 4·8 and may go down to 4·2.

METHOD OF STUDY

The method of study used was the same as that described in our former paper on the competition of barley with *H. mollis* (1947). Both barley and weed were grown in earthenware pots, 28 cm. in diameter and 25·5 cm. deep, with an upturned outlet near the bottom which enabled watering to be done without danger of loss and yet secured good aeration of the soil. The bottom of the pot was covered with coarse gravel to above the outlet. Each pot was then filled with 16·3 k. of soil taken from one of the fields at Woburn. This gives about 20 cm. depth of soil without counting the gravel at the bottom of the pot, and occupied 12·3 l. If the volume of the gravel be included as available for the plant roots, the whole space would be 14·2 l. per pot, and this remained constant during the experiments. Sufficient potassium phosphate and ammonium sulphate were added to supply all the phosphates, potash and nitrogen the plants would require. Water supply and aeration were adequate throughout the growth of the plants. The only variables were, therefore, the number of barley or *Agrostis* plants for a definite volume of soil.

We had previously shown (1947) that with the addition of 1·25 g. nitrogen per pot of the present size (or 0·09 g. nitrogen per litre of space) a full yield of barley was obtained and that more nitrogen than this simply increased the nitrogen in the plant without increasing the yield of produce. This amount was, therefore, added in two instalments, namely 0·75 g. on 17 April, just before the sowing of the competitive plants, and 0·50 g. on 3 June when the plants were vigorously growing. It was found that four plants per pot (613 sq.cm. of surface or 3·5 l. of root space per plant)

gave the maximum yield of barley, any greater thickness of sowing not causing increase of produce. This agrees closely with results obtained in previous years.

As regards the *Agrostis* plants, the thickness of planting which led to the most favourable growth was determined under similar conditions to those indicated above for barley. Portions of *Agrostis* plants from the field were planted in the soil on 11 April, at thicknesses ranging from one plant per pot (2452 sq.cm. per plant) to eight plants per pot (307 sq.cm. per plant). The details of the growth with each thickness of planting are shown in Table 1.

TABLE 1. *Effect of thickness of planting on yield of* Agrostis

No. of *Agrostis* plants per pot	Root space per plant (l.)	No. of shoots per plant on 9 June	Wt. of air-dry *Agrostis* above ground per pot (g.)	Wt. of air-dry *Agrostis* roots per pot (g.)	Nitrogen in dry produce (%)	Root space per 1 g. root (l.)
1	14·2	91	77·6	65·2	1·12	0·22
2	7·1	49	87·5	63·7	1·13	0·22
4	3·5	29	93·8	72·5	0·93	0·20
6	2·4	16	87·7	58·5	0·89	0·24
8	1·8	21	100·4	54·1	0·89	0·26

Very nearly the maximum growth per unit area is reached with four plants per pot, and little, if any, greater production of either tops or roots is obtained by thicker sowing. The weight of roots per plant throughout seems almost exactly proportional to the space available to them. In fact, it appears as if a single *Agrostis* plant is able to fill the space allotted to its roots within the first season provided it is sufficiently supplied with water and manurial constituents. There does not seem to be any stress for root space during the first year even with the thickest planting shown above.

It might be supposed that the seriousness of a twitch, as a weed in the first year, depended on the amount of the roots and the available space and available nitrogen they took up. If we compare on this basis the two twitches we have studied and the barley among which they grow, we have the figures shown in Table 2.

TABLE 2. *Comparison of* Holcus mollis *and* Agrostis gigantea *as weeds*

Results per pot	*Holcus mollis* (1944 and 1945)	*Agrostis gigantea* (1947)	Barley (1947)
Mean wt. of plants and roots (g.)	156·8	152·2	117·6
Mean wt. of parts above ground (g.)	79·7	89·4	110·8
Mean wt. of roots (g.)	77·1	62·8	6·8
Nitrogen in parts above ground (mg.)	669	815	999
Nitrogen in roots (mg.)	594	385	70
Percentage wt. roots to total produce	49	41	6
Percentage of total nitrogen in roots	47	32	7

Judged in this way, the more serious twitch is undoubtedly *Holcus mollis*, for not only does it give a smaller proportion of its total weight as possibly useful grass, but

it fixes in its roots a larger amount of the nitrogen available than the *Agrostis* we are now studying, and an enormously greater proportion than is found in a commercial cereal like barley. It would seem that one of the great merits of a commercial cereal crop is that, of the nitrogen supplied for its growth, as large a proportion as possible should be recovered in the commercial products, the grain and the straw.

COMPETITION BETWEEN BARLEY AND *AGROSTIS GIGANTEA*

So far we have dealt with the amount of barley or of *Agrostis* that can be made to grow in a limited fixed space without the competition of any other plant. We have now to consider the way in which the advent of this weed on a soil where barley is being grown will lower the yield of the barley, or, on the other hand, how far a crop of barley will be able to smother the *Agrostis*. Two cases at once arise. First, what will be the effect of an increase in the thickness of barley sowing when the weed infestation remains constant, and second, what will be the effect of an increase in the amount of weed infestation when the stand of barley remains the same? We will consider the two cases separately.

(1) *Weed infestation constant: barley thickness variable*

In all cases under this head in our experiments, there were six plants of *A. gigantea* per pot, while the number of barley plants varied from one to eight, this giving all grades of thickness from a very sparse plant of barley to a thick seeding. The *Agrostis* was transplanted from the field practically at the same time as the barley seed was put in. In all cases one effective joint of rhizome was planted to make one unit of the weed. The results are shown in Table 3.

TABLE 3. *Yield of barley and* Agrostis, *with varying amounts of barley*

No. of plants per pot		Maximum no. of barley shoots	Yield of barley per pot		Yield of weed (above ground) per pot (g.)	Wt. of roots	
Barley	Weed		Grain (g.)	Total produce (g.)		Barley (g.)	Weed (g.)
0	6	—	—	—	87·7	—	58·5
1	6	12	13·0	29·1	73·7	1·7	48·8
1	0	32	29·4	78·5	—	4·5	—
2	6	27	29·1	64·2	60·1	3·8	46·4
2	0	40	40·0	96·0	—	5·7	—
4	6	30	28·3	60·0	61·1	3·1	40·1
4	0	50	56·3	124·1	—	6·5	—
6	6	43	43·3	91·9	31·6	6·9	45·6
6	0	59	61·3	126·7	—	9·5	—
8	6	57	47·7	99·6	23·8	6·1	32·9
8	0	68	59·0	128·6	—	7·9	—

These results are in accordance with the idea that a thick seeding of a cereal crop will tend to smother the weed in this case as well as with other twitches, provided the twitch does not get a start over the barley and is a new infection. The barley and

the *Agrostis* flourished in this case, and although the thicker seeding of the barley did not altogether neutralize the effect of the weed, it did markedly reduce its influence on the yield of both grain and total produce. The actual reduction in the yield of barley (above ground) due to the presence of six *Agrostis* plants with varying thicknesses of the crop, and also the effect of the presence on the yield of *Agrostis* are shown in Table 4.

TABLE 4. *Reduction in yield of barley owing to presence of* Agrostis *and of* Agrostis *owing to presence of barley*

No. of plants per pot		Reduction in yield		
		Barley		*Agrostis*
Barley	*Agrostis*	Grain (%)	Total produce (%)	Total produce (%)
1	6	56	63	16
2	6	27	33	31
4	6	50	52	30
6	6	29	27	64
8	6	19	23	73

Despite some irregularity in the case of four plants of barley, the general results are quite clear. The increased thickness of planting of the barley makes a great difference to the competitive power of the crop over the weed. With the highest thickness of barley (eight plants per pot or 307 sq.cm. of surface per plant) the reduction in yield by the presence of six plants of *Agrostis* per pot (409 sq.cm. of surface per plant) is only about 20% whether judged by the yield of grain or of total produce. On the other hand, this thickness of barley has reduced the yield of *Agrostis* above ground by 73%.

Table 5 shows how *A. gigantea* compares as a competitive weed with the *Holcus mollis* previously studied.

TABLE 5. *Comparison of* Holcus mollis *and* Agrostis gigantea *as competitive weeds in barley*

No. of plants per pot			Reduction in total produce			
			Holcus mollis		*Agrostis gigantea*	
Barley	Weed		Reduction in barley (%)	Reduction in weed (%)	Reduction in barley (%)	Reduction in weed (%)
1	6		68	26	63	16
2	6		57	31	33	31
4	6		42	49	52	30
6	6		29	61	27	64
8	6		33	56	23	73
		Mean	46	45	40	43

These figures suggest that the *Agrostis* under study is slightly less effective as

a competitor with barley than was the *Holcus* previously examined. With the greatest density of barley growth, the relative reduction in the crop was, with *Holcus*, 33% and with *Agrostis*, 23%. The twitch itself is reduced by the barley by 56% with *Holcus*, and by 73% in the case of *Agrostis*.

(2) *Barley plant constant: weed infestation varying*

We may now examine the result of increasing the number of weed units on the vigour and development of a sparse barley crop. To do this, a fixed number of two barley plants per pot were sown, thus allowing 7·1 l. of root space per plant. The number of *Agrostis* plants varied from 0 to 8, that is to say, from a pot completely free from the weed to one thickly planted. As before, the barley and the weed were planted at the same time, so that neither got any substantial start over the other. The results are shown in Table 6.

TABLE 6. *Yield of barley and* Agrostis, *with varying amounts of weed*

No. of plants per pot		Maximum no. of barley shoots	Yield of barley per pot		Yield of weed (above ground) per pot (g.)	Wt. of roots	
			Grain (g.)	Total produce (g.)		Barley (g.)	Weed (g.)
Barley	Weed						
2	0	40	40·0	96·0	—	5·7	—
2	1	38	43·1	97·2	26·0	5·8	19·2
0	1	—	—	—	77·6	—	65·2
2	2	35	34·7	83·4	36·2	4·9	27·8
0	2	—	—	—	87·5	—	63·7
2	4	29	32·5	70·3	57·5	4·2	32·8
0	4	—	—	—	93·8	—	72·5
2	6	24	25·1	54·4	65·8	3·2	42·5
0	6	—	—	—	87·7	—	58·5
2	8	22	21·5	45·9	65·6	2·7	47·3
0	8	—	—	—	100·4	—	54·1

The effect of the weed is very substantial, even when it is in relatively small amount compared with the barley. Table 7 shows how this effect increases with the proportion of twitch to barley.

TABLE 7. *Effect of increasing proportion of* Agrostis *on yield of both weed and barley*

No. of plants per pot		Wt. total produce of barley (with roots) (g.)	Percentage of wt. of barley grown alone	Wt. *Agrostis* (with roots) (g.)	Percentage increase on wt. with 1 weed plant
Barley	Weed				
2	0	101·7	100	—	—
2	1	103·0	101	45·2	100
2	2	88·3	87	64·0	142
2	4	74·5	73	90·3	200
2	6	57·6	57	108·3	240
2	8	48·6	48	112·9	250

The effect of a small amount of twitch among the barley appears negligible, but as the amount increases so the reduction in barley yield rapidly grows, though such reduction is never so great in proportion as the increase in the weight of *Agrostis* produced. The presence of two plants of barley in a space of 14·2 l. has been able to lower the production of one plant of *Agrostis* to 32% of that obtained in the absence of barley. In this case it is not great pressure on root space which is the cause of the reduction, for with a larger number of weed plants the root production is much greater. A similar result emerged from our study of *Holcus mollis*, and our experience in the two cases seems to suggest that there is some preventive influence exerted on the twitches by the presence of even a sparse barley plant. Even when the number of *Agrostis* plants is four times as great as that of barley, the latter was still able to reduce the growth of the weed to 73% of that when it was grown alone. In this case, however, it seems probable that this was at least partly due to pressure on root space.

If the seriousness of *Agrostis* as a weed is compared with that of *Holcus mollis* in the same soil, these figures again illustrate the fact that the latter is a more powerful competitor with barley than is the former. The reduction in yield of barley by a definite number of twitch plants is about 20% greater with *Holcus* than with *Agrostis*, and this at almost every degree of infestation. Apart from this, the behaviour of the two weeds is very similar, and it is only when the number of *Agrostis* plants exceeds that of the barley that any very serious reduction in the yield of the latter takes place, always on the assumption that the twitch does not get a start before the barley is sown.

The way in which the available nitrogen absorbed by the plants distributes itself between barley and *Agrostis* when the former is in constant amount may be seen in the figures given in Table 8.

TABLE 8. *Partition of nitrogen taken up in mixed cultures of barley and* Agrostis gigantea

No. of plants per pot		Amount of nitrogen in barley (with roots) (g.)	Amount of nitrogen in *Agrostis* (with roots) (g.)	Percentage of nitrogen recovered in	
Barley	Weed			Barley	*Agrostis*
2	0	1021	—	100	—
2	1	776	337	70	30
2	2	665	473	58	42
2	4	528	667	44	56
2	6	407	772	34	66
2	8	335	851	28	72

Of the two competing plants, the barley is thus able to secure a little more of the nitrogen taken up than its proportion in the mixture would justify. Where there was an equal number of plants of barley and of weed, the barley obtained 58% of the nitrogen taken up, and similarly all through, in spite of the fact that the root

development of the barley was much the smaller of the two. A very similar relationship between the nitrogen absorbed by the competing plants was found with barley and *Holcus mollis*.

ROOT INTERACTIONS BETWEEN BARLEY AND *AGROSTIS GIGANTEA*

As far as can be seen by eye examination alone, there is no sign of any repulsion or attraction between the roots of the two competing plants. They were, in fact, as was the case with *H. mollis*, simply intermingled in a thick network of root fibres. But it has been recently suggested (Ahlgren & Aamodt, 1939) that there are interactions between the roots of a number of grasses which cannot be accounted for on the basis of a differential response to light, temperature, moisture, manuring, and management, and the present study may be expected to throw some light on the

TABLE 9. *Interactions between barley and the two weed plants*

	Total yield above ground	
	Agrostis gigantea (1947) per pot	*Holcus mollis* (1945) per pot
I. Yield of 2 barley plants alone and with 2 additional barley or weeds:		
(*a*) 2 barley plants alone (g.)	96·0	156·5
(*b*) 2 barley plants with 2 additional barley (g.)	62·1	76·2
(*c*) 2 barley plants with 2 weed plants (g.)	83·4	116·1
Reduction in yield of 2 barley plants:		
(1) Reduction of *b* on *a* (%)	35	52
(2) Reduction of *c* on *a* (%)	13	26
II. Yield of 2 weed plants alone and with 2 additional weed or barley plants:		
(*a*) 2 weed plants alone (g.)	87·5	107·7
(*b*) 2 weed plants with 2 barley plants (g.)	36·2	43·1
(*c*) 2 weed plants with 2 additional weeds (g.)	46·9	63·6
Reduction in yield of 2 weed plants:		
(1) Reduction of *b* on *a* (%)	59	52
(2) Reduction of *c* on *a* (%)	46	26
III. Yield of 2 barley plants alone and with 4 additional barley or weeds:		
(*a*) 2 barley plants alone (g.)	96·0	156·5
(*b*) 2 barley plants with 4 additional barley (g.)	42·2	54·6
(*c*) 2 barley plants with 4 weed plants (g.)	70·3	82·0
Reduction in yield of 2 barley plants:		
(1) Reduction of *b* on *a* (%)	56	65
(2) Reduction of *c* on *a* (%)	27	48
IV. Yield of 4 weed plants alone and with 2 additional weed or barley plants:		
(*a*) 4 weed plants alone (g.)	93·8	127·1
(*b*) 4 weed plants with 2 barley plants (g.)	57·5	67·0
(*c*) 4 weed plants with 2 additional weeds (g.)	58·4	75·8
Reduction in yield of 4 weed plants:		
(1) Reduction of *b* on *a* (%)	39	47
(2) Reduction of *c* on *a* (%)	38	40

matter. The figures given in Table 4 show that the effect of a few plants of barley on the amount of *Agrostis* growth is great. We may now extend this evidence, and in Table 9 we have given the results both for *A. gigantea* and also *Holcus mollis* in two typical cases, namely where there are two plants of barley with and without either two or four plants respectively of each of the weeds, in the case where the crop and the weeds start their growth at approximately the same time.

From these figures the following conclusions can be drawn:

(1) An increase in the thickness of barley planting affects the yield per plant of that crop more than an equal increase of plant units of either *Holcus mollis* or *Agrostis gigantea*.

(2) *Holcus mollis* affects barley more than *Agrostis gigantea*, i.e. it is a more powerful competitor with it.

(3) Barley has a greater effect on the growth of either of the weeds than has a similar increase in the number of units of the weed itself. The difference is not very great when the weed is large in proportion to the barley.

(4) The injurious influence of one grass on another, apart from any effect of water, plant food, etc., seems to be proved, and this effect varies from one grass to another, and seems to be a specific property of each grass.

REFERENCES

Ahlgren, H. L. & Aamodt, O. S. (1939). Harmful root interactions as a possible explanation for effects noted between various species of grasses and legumes. *J. Amer. Soc. Agron.* **31**, 982.

Brenchley, W. E. (1920). *Weeds of Farm Land*, pp. 126, 151 and 174. London.

Donald, C. M. (1946). Competition between pasture species with reference to the hypothesis of harmful root interactions. *J. Coun. sci. indust. Res. Aust.* **19**, 32.

Mann, H. H. & Barnes, T. W. (1947). The competition between barley and certain weeds under controlled conditions. II. Competition with *Holcus mollis*. *Ann. appl. Biol.* **34**, 252.

Philipson, W. R. (1937). A revision of the British species of *Agrostis*. *J. Linn. Soc.* (*Bot.*), **51**, 73.

Sinclair, G. (1824). *Hortus Gramineus Woburnensis*, p. 344. London.

(*Received* 20 *September* 1948)

PROCEEDINGS OF THE ASSOCIATION OF APPLIED BIOLOGISTS

Ordinary Meeting of the Association held on Friday, 3 December 1948, in the Imperial College of Science and Technology, London; the President, Mr W. C. Moore, in the Chair.

Symposium on current research methods observed in U.S.A.

The following papers were read and discussed:

Some impressions of a nematologist. By Dr T. Goodey.

Recent American work on insect-transmitted virus diseases. By Mrs M. A. Watson.

Plant physiology in agricultural research in America. By Dr D. J. Watson.

REPORT OF THE COUNCIL OF THE ASSOCIATION OF APPLIED BIOLOGISTS FOR THE YEAR 1948

The Officers and Council of the Association were as follows:

President: W. C. Moore, M.A.
Vice-Presidents: C. B. Williams, M.A., Sc.D.; I. Thomas, M.Sc., Ph.D.
Hon. Treasurer: H. F. Barnes, M.A., Ph.D.
Hon. Secretary: G. V. B. Herford, O.B.E., B.A., M.Sc.
Hon. Asst. Secretary: J. R. Busvine, B.Sc., Ph.D.
Hon. Editor of The Annals of Applied Biology: R. W. Marsh, M.A.
Hon. Asst. Editor: I. Thomas, M.Sc., Ph.D.

Council: H. J. Craufurd-Benson, M.B.E., Ph.D.; C. Ellenby, D.Sc.; J. A. Freeman, Ph.D.; H. H. Glasscock, M.Sc.; Miss M. D. Glynne, D.Sc.; F. H. Jacob, M.Sc.; D. Price Jones, Ph.D.; W. G. Keyworth, Ph.D.; A. J. Musgrave, M.Sc.; D. Snow, Ph.D.; F. J. D. Thomas, B.Sc.; R. L. Wain, Ph.D.

The Council is able to report another year of progress for the Association. The membership has continued to increase, 55 new members having been elected during the year. For the first time Ordinary Membership has been opened to applied biologists throughout the world of any nationality. As a result about six non-British subjects have been elected Ordinary Members. The total membership, including 11 Honorary Members, now stands (31 December 1948) at 530, an increase of 44 over the previous year's total which itself was a new high figure.

It is with deep regret that the Council have to record the deaths of Sir John Fryer, Dr G. H. Pethybridge and Dr A. Smith. Sir John had been a member of the Association since 1913, being Hon. Treasurer 1914–19, on the Council 1921 and 1924–5 and President 1926–7. Dr Pethybridge was one of the two surviving original members and had been on the Council 1912–13 and 1925–7 and Vice-President 1926–7. Dr Smith was assistant mycologist at the Ministry of Agriculture's Plant Pathology Laboratory, Harpenden.

Six paper-reading meetings were held in London during 1948, an average of 77 members and visitors recording their names as being present on each occasion.

The Summer Meeting, held on 11 June, consisted of a visit to the Commonwealth Mycological Institute and the Royal Botanical Gardens, Kew, by kind invitation of the Directors of the two institutions.

A second Conversazione was held in the Botany Department, Imperial College of Science on 23 July, to which delegates from the Commonwealth Entomological and Mycological Conferences were invited. There was an even larger attendance than at the similar function held last year.

The following papers were presented to the Association during the year:

20 February. J. A. Freeman: Stored product pests: a survey of the principal entomological problems. E. E. Turtle: Stored product pests: a survey of the development and application of methods of control.

22 March. Symposium on biological aspects of water pollution. B. A. Southgate: Introduction. L. A. Allen: Some microbiological problems in the treatment of sewage and trade waste. T. G. Tomlinson: The biology of a percolating filter. Ll. Lloyd: Insect destruction of the filter film and a compensating factor. F. T. K. Pentelow: The effect of pollution on fisheries.

23 April. R. J. Bray and P. A. Wells: The organization of wool research and education. These papers were followed by three films entitled Blowfly strike, Control of worms in sheep and Shearing at Big Billabong. This was the first occasion on which music was heard at an Association paper-reading meeting. A. B. Wildman: Problems of wool growth. J. Barritt: The protection of wool against attack by insect pests.

1 October. Symposium on nutrition in relation to plant disease. F. C. Bawden: Some effects of host nutrition on susceptibility to plant viruses. M. H. Moore: Some fruit diseases. W. G. Keyworth: Experiments on the relationship of plant nutrition to Verticillium wilt of the hop. N. H. Pizer: Hop Verticillium wilt, some problems and difficulties.

5 November. Symposium on the insecticidal properties of certain organo-phosphorus compounds. H. Martin: Introduction. H. E. Coates: The chemistry of phosphorus insecticides. S. H. Bennett: Some preliminary experiments with systemic insecticides.

3 December. T. Goodey: Some impressions of a nematologist (visiting America). D. J. Watson: Plant physiology in agricultural research in America. Mrs M. A. Watson: Recent American work on insect-transmitted virus diseases.

Two Special General Meetings have been held during the year on 20 February and 5 November to consider proposed alterations to Rules 4, 5, 8 and 14. As a result Ordinary Membership of the Association has been extended beyond the confines of British citizenship and Honorary Membership of the Association is now open to British subjects. A slight increase has been made in the Entrance Fee, i.e. from 1 January 1949 it is half the annual subscription instead of being 10*s.* 6*d.* In addition, any member, after twenty-five years' membership may, at or after the age of sixty, pay an annual subscription of 12*s.* 6*d.* or may compound for all future subscriptions by a payment equal to five full annual subscriptions.

The Council wishes to take this opportunity of expressing its thanks to the authorities of the Imperial College of Science and Technology for so generously affording the Association accommodation for its meetings.

The Hon. Editor represented the Association at the Scientific Information Conference called by the Royal Society. The Hon. Secretary and Hon. Treasurer were the official delegates of the Association to the VIII International Congress of Entomology held at Stockholm during August. Dr J. T. Martin is the representative of the Association on the Executive Committee of the Second International Congress on Plant Protection to be held in London during 1949.

The Council wishes to place on record its warm appreciation of the legacy of £100 free of duty made by the late Dr G. H. Pethybridge to the Association to be used for the promotion of scientific research and for the publication of the results thereof.

HON. EDITOR'S REPORT FOR 1948

Vol. **35** of the *Annals of Applied Biology* consisted of pp. xi+652 and 7 Plates, as against pp. xi+651 and 16 Plates for vol. **34**. Including papers published in *Proceedings*, vol. **35** contained 60 communications, 53 being by Members of the Association. The subjects may roughly be classified as follows: general applied botany, 11; mycology and fungus diseases, 7; viruses and virus diseases, 9; general applied zoology, 9; entomology and insect pests, 9; applied microbiology, 2; insecticides and fungicides, 13.

To meet the continuing rise in demand for the *Annals*, Council agreed at the end of 1948 to increase the subsequent printing order to 1450 copies. This is more than double the 1944 figure.

The possibilities of reducing publication delays were fully discussed in July with the University Printer and members of the staff of the Cambridge University Press. It was agreed to dispense with the preliminary preparation of estimates and to send in scripts at earlier dates. These changes affected only the last number of the volume and the dates of issue of the four parts were: no. 1, 26 June; no. 2, 17 August; no. 3, 11 December; no. 4, 25 January.

The Editor helped to represent the Association at the Royal Society's Scientific Information Conference, 21 June–2 July, and acted as Recorder of Working Party i/c dealing with subject grouping in existing periodicals. The full report of the Conference has been received from the Royal Society for transmission to the Association. It may here be noted that the Conference offered no criticism of the practice of publishing, in one journal, papers on a number of subjects covering the range of interests of members of the publishing Society. The Conference did, however, suggest that by friendly consultation between editors some improvement in the allocation of papers between biological journals might be effected. The valuable services performed by the Biological Council in providing for joint meetings of editors were noted.

The Editor again expresses his sincere thanks to the Assistant Editor and to the members of the Publications Committee; in particular to Dr J. W. Evans, who is now retiring from the Committee after four years' service. Special reference must also be made to the part played by Mr D. J. Finney. Mr Finney's help is indeed indispensable in the present phase of biological research, when the rapid development of statistical methods and technique is such an outstanding feature.

PLANT PESTS AND DISEASES COMMITTEE REPORT FOR 1948

Two meetings have been held during the session, one on 18 March and the other on 6 January 1949. S. H. Crowdy, W. P. K. Findlay, C. G. Johnson, S. W. Rolfe and A. R. Wilson were appointed by the Council to fill the vacancies caused by the retirement under the rules of W. G. Keyworth, J. T. Martin, M. H. Moore and I. Thomas and the retirement from the Committee of A. C. Evans to take up an appointment abroad. At the first meeting P. H. Gregory was elected Chairman and S. H. Bennett was elected Secretary.

The Sub-Committee formed in collaboration with the Ministry of Agriculture Conference of Advisory Entomologists has continued its work in the compilation of the list of common and scientific names of British insect and other pests. Thysanoptera, Aphaniptera and Ixodidae have been completed.

On the training of helminthologists in Great Britain it is hoped that the first training and revision course will take place during the summer of 1949.

The Committee has continued to assist the Plant Pathology Committee of the British Mycological Society in the definition of terms used in plant pathology. It has just formed a Sub-Committee to prepare a list of definitions of terms used in Economic Entomology. (The term Economic Entomology to include Helminthes and other groups that come within the scope of the economic entomologist.)

The Committee has considered the nomenclature of economic plants and pathogenic fungi. A report is being prepared for Council with suggestions of the course of action that Council should take before the next International Botanical Congress at Stockholm in 1950 to facilitate the retention of well-known names.

The Committee held a full-day informal conference to discuss the problems of spray damage. To this conference were invited a number of plant physiologists together with chemists, entomologists and mycologists who had had considerable experience of the problem. The conference held on 7 January produced a very fruitful discussion. A report on the conference is now being prepared.

In view of the considerable difficulty being experienced with the various systems employed by workers in the nomenclature and classification of viruses, the Committee at its last meeting formed a small Sub-Committee to consider the existing systems of nomenclature and classification of viruses and to recommend which system is best suited for this country.

REVIEWS

The Filterable Viruses (*Virales*). By F. O. Holmes. Pp. 159+xxiii. Baltimore: The Williams and Wilkins Co. $2.50.

It is generally agreed, and has been for many years, that the naming of viruses is deplorably chaotic and that 'something should be done about it'. That there is less agreement about what should be done is hardly surprising, for their position in the evolutionary scale is far from established and it is debatable whether viruses would be better suited by names similar to those used for organisms or by some quite different type. Nevertheless, most workers would probably welcome a nomenclature based on some system of classification and reflecting degrees of natural relationships. Realizing this, Dr F. O. Holmes in 1939 produced his *Handbook of Phytopathogenic Viruses* in which he introduced a Latin binomial-trinomial system of naming, based on a proposed classification in which the viruses of seed plants were separated into ten monogeneric families. Some workers, particularly in the U.S.A., have used this new nomenclature, but, despite the attractive familiarity of its form, it has not been generally adopted. Now, however, it seems to be gaining a wider acceptance, for in 1948 a revised version, extended to include animal viruses as well as those attacking seed plants and bacteria, appeared as a supplement to the Sixth Edition of *Bergey's Manual of Determinative Bacteriology*; with an enlarged index, but without change of page numbers, the supplement has now been published separately under the title *The Filterable Viruses*.

Dr Holmes's desire to bring order out of chaos is one that is entirely laudable; praiseworthy, too, is the immense industry he has shown in renaming, and summarizing data on, 46 bacteriophages, 128 plant viruses and 66 viruses of man and animals. He has produced a useful reference book, with a valuable collection of selected references to original papers and much information in commendably few words, although in places the brevity confers an air of certainty that some statements hardly warrant. Such features, however, are incidental to the main purpose of the book, which is to establish a system of classifying and naming viruses. It is on this that it must be judged, and to this end it is pertinent to ask some questions. Is the scheme likely to provide a basis for a lasting scheme of classification? Are viruses grouped and separated according to their various degrees of similarities and differences? Are groups homogeneous? Do names indicate natural relationships? To these, and other such questions that might be asked, the only possible reply is 'No', and the more the scheme is contemplated, the more unqualified the 'No' becomes.

Close inspection shows that the scheme is far less of an innovation than it seems at first sight, for no new taxonomic criteria are used. Symptomatology, aided by methods of transmission, provides the basis for grouping, although its fallibility is notorious. Indeed, it is largely because workers have had unjustified faith in the diagnostic value of host reactions that the plague of synonymity has arisen in the past. There is no reason to think that such extrinsic properties reflect structural similarities; on the contrary, it is well known that one virus can cause symptoms of quite distinct types in different hosts and that related strains of one virus may cause different diseases in one and the same host. Classification, if it is to reveal natural relationships, must be based on intrinsic characters of the objects being classified, and not on features shown through others. Differences in pathogenicity are invaluable in showing that two viruses which otherwise seem similar are not identical, but they are most insecure criteria for making major taxonomic decisions.

In deriving some of his proposed names, Dr Holmes has shown considerable ingenuity, but many are merely latinized versions of old symptom names. Translating tobacco mosaic and cucumber mosaic into *Marmor tabaci* and *M. cucumeris* does not automatically classify them into species of one genus. It merely creates a false air of having done so, and suggests similarities that there is every reason to consider do not exist. Some of the suggested families and genera may be homogeneous, but, if so, they are those such as *Chlorogenus* (viruses

causing yellows diseases) about whose intrinsic properties we know little or nothing. The viruses whose properties have been studied at all intensively are grouped and separated on arbitrary and taxonomically invalid bases, and the new names of many imply relationships that are palpably false. At present it must be admitted that most viruses are unclassifiable, because too little is known about their structure and fundamental properties. The most that can safely be done is to group related strains around named types, which it would seem might be achieved by extending studies on serological relationships and mutual antagonism, and arrangements into groups analogous to genera and families must wait until suitable criteria are forthcoming. There is no reason to assume that this delay would create any great problems, for the number of plant viruses is not so large that a determinative system of classification is needed immediately. The present confusion has arisen because the same virus, or related strains of one virus, have been described under many different synonyms. Let us first sweep these away and clear the lines at the species level, when advances towards grouping at higher levels may seem less formidable than now. To achieve this, workers will need to consider taxonomic relationships much more than in the past and, in describing 'new' viruses, they must look for other reasons than a previously undescribed symptom. If Dr Holmes's book persuades virus workers of the need to consider relationships more than has hitherto been customary, it will have fulfilled a valuable task. It is to be hoped, however, that his scheme will not achieve wide or official acceptance, for this would not be in the best interests of virus research. The use of symptoms for purposes of classification has a tempting facility, but it can never be more than a temporary makeshift and it could be adopted for no better reason than despair of ever obtaining adequate data for true taxonomic groupings.

F. C. BAWDEN

Flax Retting with Aeration. Pp. vi and 146. Water Pollution Research Technical Paper No. 10, published for the Department of Scientific and Industrial Research by H.M. Stationery Office. 3*s*.

This volume is a success story. Setting out originally to solve an effluent problem of flax retting, the Water Pollution Research Laboratory not only solved the problem by retting with aeration, but also worked out the conditions for the practical operation of this method of retting.

Flax cultivation was non-existent in Great Britain at the beginning of the second World War except for experiments at a branch of the Linen Industry Research Association on the Royal Estate at Sandringham. Soon after war broke out in 1939 this branch was taken over by the Government, and its nucleus stocks were multiplied until in 1944 over 60,000 acres were under flax, and seventeen factories had been erected to process it.

For ease of operation and to avoid the difficulties of effluent disposal, the flax fibre was mostly separated as green flax, i.e. without retting. But green flax is less useful than retted flax and less popular with spinners, and in 1941 the Water Pollution Research Laboratory was asked by the Ministry of Supply to assist in dealing with the problem of disposal of the waste waters produced in the retting process. These waters contain much organic matter, including organic acids, and special means of treating them were found to be essential at twelve of the seventeen Ministry of Supply factories.

Technical Paper No. 10 describes the various lines of work undertaken with the aim of continually re-using the waste waters for further rets. The investigation, as would be expected from the Water Pollution Research Laboratory, was a most comprehensive one, and is fully described in the twelve chapters and three appendices of the paper: full details of the results are given in seventy tables.

In the initial experiments the methods used were similar to those used for treating sewage, including circulation of the retting liquor through a biological filter and circulation of the liquor through an aeration tank. Batches of flax were retted at the same time by the usual anaerobic process to serve as controls. Detailed chemical and bacteriological examinations

were made of the flax during retting. The dried retted flax was conditioned, scutched on a commercial machine, examined by a grader, and, in selected samples, subjected to spinning tests to see whether the flax quality compared favourably with the controls.

The results of small-scale rets showed that the use of percolating filters would entail considerable cost and that the treatment of waste water with coagulants would not be satisfactory for a full-scale rettery. The results of the small-scale rets with circulation of retting liquor through a separate aeration tank were more promising and proved satisfactory on the full scale, but finally the simpler method was preferred of retting with aeration of the retting liquor in the retting tank itself. Such liquor can thus be successfully re-used any number of times throughout the retting season.

The investigation is an excellent object lesson in the energetic attack of a difficult practical problem along a number of lines of approach simultaneously, with ultimate success being achieved along one of them in approximately two years. By the time the war ended the process was well established at a number of the flax factories, which were thus enabled to continue functioning by producing a type of flax for which there is always a demand in days of peace. A. J. TURNER

A Catalogue of Insecticides and Fungicides. Vol. I: *Chemical Insecticides*; Vol. II: *Chemical Fungicides and Plant Insecticides*. By DONALD E. H. FREAR. Waltham, Mass.: Chronica Botanica Co.; London: Wm. Dawson and Sons Ltd. Vol. I: pp. 203, 1947, $6.50; vol. II: pp. 153, 1948, $5.50.

Project 999, undertaken during the second World War by the Pennsylvania Agricultural Experiment Station, aimed at the discovery of new insecticides and fungicides to replace those made scarce by war. Part of this work was the collection of all previously published records of toxicity tests of chemicals and plant materials and, in a letter to *Science*, an appeal was made for unpublished data on this subject. The assemblage and correlation of the enormous mass of information so obtained fell to Dr Donald E. H. Frear who has now completed records to January 1944. Unexhausted by the magnitude of his task, he again asks for further information of published and unpublished work in a field which has extended to regions undreamt of in 1944. The territories of the chlorinated hydrocarbons and of the organo-phosphorus compounds had then only just been discovered, and these two fields alone would already add another full volume to the catalogue.

The first difficulty faced was the arrangement and classification of the chemical compounds tested, for which purpose a special coding system was devised. But the problem of chemical coding still remains. If other suggestions such as the systems devised by Dyson or by Gordon and his colleagues receive general acceptance, Dr Frear's task will be simplified.

A second difficulty, one of testing rather than of cataloguing, is the selectivity in toxic action which renders testing against a limited range of insect species a hit-or-miss affair. Dr Frear has a concise way of giving available data, for example: 'Azobenzene (code number 665–952): $C_6H_5N{:}NC_6H_5$. *HT* screw worms and *HT* greenhouse red spider at 1%; *T* codling moth and mosquito larvae; *NT* southern army worm at 4%. 156, 488, 915, 1481.' The four numbers lead the reader to the appropriate references though unfortunately 1481 is missing from the index.

The catalogue is a fine tribute to Dr Frear's industry and patience and will be most useful to all interested in the chemistry of insecticides and fungicides. H.M.

The Principles of Biological Assay. By C. W. EMMENS. Pp. 206 + xv. London: Chapman and Hall Ltd. 1948. 21*s*.

The application of statistical techniques to the analysis of experimental data is most often undertaken for the purpose of detecting and estimating the effects of applied treatments. In biological assay, the objective is rather the estimation of causes from measurement of the effects they produce. Most of the familiar topics of statistical science—analysis of variance,

regression, estimation of parameters, efficient experimental design—are encountered, but the emphasis on them is a little different when the purpose of statistical analysis is the derivation of an estimate of potency rather than the comparison of treatment effects. In this first book to be devoted to the statistical aspects of biological assay, Dr Emmens presents an admirable account of basic principles, assay design, and methods of analysis, in a manner that may be understood by readers whose previous knowledge of statistics is slight. The author's personal experience of the practice of biological assay ensures that the techniques he describes are those of chief practical importance, but there is no attempt to conceal the difficulties of the subject by a pretence that the arithmetical rules can be easily learnt and applied without an understanding of the fundamental issues involved.

The first half of the book is an introduction to statistical methods, with special attention given to regression. Valuable as this is for the reader who has no previous knowledge of statistics, some may regret that Dr Emmens did not content himself with references to standard texts in order that he might have more space for the full development of his main theme. Four chapters on assays based on parallel dose-response lines show details of calculations for testing the validity of assays as well as for forming potency estimates. The advantages to be gained from symmetrical designs are emphasized and the analyses of types suitable for special purposes are described. The inter-relationship between design, scheme of randomization, and analysis is made very clear; for example, the reader is cautioned against neglect of inter-cage variation, and is shown how the particular method adopted in order to eliminate its effect influences the form of the statistical analysis. Assays based on quantal responses, and their analysis by means of the probit transformation are discussed in the next three chapters. Further chapters are concerned with the use of reaction time as a response, the simultaneous assay of several preparations and the combination of results of different assays, and the choice of a response metameter: under this last heading, the common assumption that a combination of initial and final measurements on the same animal is always preferable to the final alone is challenged, and the use of covariance methods (illustrated earlier) for determination of the most sensitive measure of response is recommended. A final chapter describes, briefly but clearly, assays of the slope-ratio type. It is surprising to find no mention of the use of non-linear dose-response regressions in assay work, particularly as Dr Emmens has himself published a valuable and interesting study of the application of logistic curves for this purpose.

That a reviewer who is himself deeply interested in the subject of this book should find some grounds for adverse criticism is scarcely surprising. To regard the fitting of a probit regression line as an example of 'least squares' is misleading, since the basis of the method is the maximizing of the likelihood, not the minimizing of χ^2. Calculation of expected probits to three decimals, and the resultant interpolation in order to obtain 'corrected' (a better term is 'working') probits is surely unnecessary unless large batches of subjects have been tested. The numerical examples of probit calculations do not seem to be ideal computational patterns, as excessive numbers of decimal places and unwanted columns have been included. The account of the fitting of probit planes seems irrelevant to assay problems, and indeed the whole chapter on reaction times—admittedly an awkward topic—is perhaps the least satisfactory in the book. An account of the simultaneous assay of three samples of penicillin is marred by the admission that the cylinders were not assigned at random to the doses; the comment that the sensitivity of the assay is thereby impaired is far from clear, but seems a serious understatement of a criticism that may destroy the validity of all assessment of precision.

These are minor flaws. Dr Emmens has written a book that is sure of a welcome in every laboratory where any form of biological assay is practised. The chief regret of the reviewer is that the book is not considerably longer. In spite of the disclaimer on the dust cover, it is by no means without value as a general text-book on statistical science. That is a minor function: more important is the fact that, as must be agreed by all concerned in the propagation of statistical methodology, the first book in this field is both sound in principle and clear in presentation, two features that will ensure its remaining in favour even when its success leads to the appearance of rivals.

D. J. FINNEY

LAWS OF THE ASSOCIATION OF APPLIED BIOLOGISTS

I. The Association shall be called 'The Association of Applied Biologists'.

II. The object of the Association shall be to promote the study and advancement of all branches of Biology with special reference to their applied aspects.

III. The Association shall consist of Ordinary and Honorary Members.

IV. Each candidate for ordinary membership shall be suitably qualified. The nomination form of each candidate for ordinary membership shall bear the signatures of two members and shall be forwarded to the Secretaries. The nomination shall be submitted to the Council, and, if approved, the election of the candidate shall be recommended to the Association at the next General Meeting. For the election of any candidate two-thirds of the votes of the Members present and voting shall be cast in favour of the candidate.

V. All ordinary members on first election shall pay an entrance fee equal to one-half the annual subscription. The annual subscription is due on 1 January each year. Ordinary Members shall pay an annual subscription of 25*s*.

Any member may compound for his subscription by payment of a sum equal to twenty annual subscriptions. Any member, after twenty-five years' membership may, at or after the age of sixty, pay an annual subscription of 12*s*. 6*d*. or may compound for all future subscriptions by a payment equal to five full annual subscriptions.

VI. Every Member elected to the Association shall receive notice to that effect from the Secretaries and shall continue a Member until his written resignation shall be received by the Secretaries, or until his membership be forfeited under the laws. (A Member shall be liable for the annual subscription for the year in which his resignation takes effect and, notwithstanding resignation, shall, if he so desires, receive any subsequent publications of the Association issued during that year.)

VII. Ordinary Members shall be entitled to admission to all the meetings of the Association, to vote thereat, to present papers, to take part in discussions, and to receive a copy of the Association's publications. Each Member shall be entitled personally to introduce non-members to any General Meeting of the Association. But no Member whose subscription is in arrears shall be entitled to vote at a General Meeting or to receive the Association's publications, nor shall any publications be sent to a new Member until his entrance fee and subscription shall have been received.

The Council may remove from the roll of the Association any Member whose subscription is one year or more in arrears.

VIII. Honorary Members shall be persons who have contributed to an eminent degree to the advancement of the Science of Applied Biology or who have rendered exceptionally meritorious service to the Association. They shall be recommended by a majority of the whole Council and elected in the same manner as Ordinary Members. The number of Honorary Members shall not exceed fifteen and not more than two shall be elected in any one year. Honorary Members shall each receive a copy of the Association's publications and shall not be liable for payment of an entrance fee or annual subscription. Their privileges shall be the same as those of Ordinary Members.

IX. The business of the Association shall be conducted by a Council consisting of a President, a Treasurer, two Secretaries, an Editor and an Assistant Editor of the *Annals*, and twelve Ordinary Members, four of whom shall retire each year and shall not be eligible for re-election within one year. The retiring President may be invited to serve as an additional

member of Council and as a Vice-President for a period of two years. One other member of Council shall be nominated by the President to act as a Vice-President.

X. All properties of the Association, both present and future, shall be deemed to be vested in the Council of the Association for the time being, in conformity with the provisions of the Literary and Scientific Institutions Act, 1854.

XI. The Council shall meet at such times as they may determine; six members shall form a quorum.

XII. The Council shall have the power to fill any vacancies among its number that may occur other than those resulting from the selection for annual retirement from the Council referred to in Law XVII.

XIII. The Council shall have power, at any of their meetings, by two-thirds of the votes of those present and voting, to recommend the removal from the roll of membership of the name of any Member for the reason that in their opinion it is contrary to the interests of the Association that he shall remain a Member. Such recommendation shall be submitted to the Association at the next General Meeting. For the ejection from the Association of any Member two-thirds of the votes of the Members present and voting shall be cast in favour of such ejection.

XIV. The Council shall appoint an Editorial Committee consisting of not more than four members to assist the Editors in the publication of the journal of the Association.

XV. The Council, at a meeting prior to the Annual General Meeting, shall appoint one or more Auditors to audit the Treasurer's accounts.

XVI. The Council shall purchase such books, instruments, specimens, furniture and other necessaries as may be required, pass the accounts and authorize their payment, and generally manage the affairs and administer the funds of the Association.

XVII. At a Council Meeting not less than one month prior to the Annual General Meeting, the Council shall nominate a President, a Treasurer, two Secretaries, and an Editor and an Assistant Editor of the *Annals*. At least three weeks before this Council meeting a notice shall be sent to each Member resident in the British Isles giving the names of the four retiring members of Council and asking for nominations, duly seconded, to fill the four vacancies. These nominations shall be included with any put forward by the Council. The list of nominations for Officers and ordinary members of Council shall be sent to all Members resident in the British Isles, at least two weeks before the date of the Annual General Meeting.

The election of Officers and ordinary members of Council shall be conducted in the following manner: At the Annual General Meeting the list of nominations for Officers and for the Council vacancies shall be read. If the number of persons proposed does not exceed the number to be elected the list shall be put to the meeting and voted on by a show of hands and the result declared by the Chairman. If there are more names than positions to be filled a ballot shall be taken. The Chairman shall appoint from among the Members present two persons, not candidates for election, to act as Scrutineers. Each Member voting shall hand in person to one of the scrutineers a copy of the list on which has been indicated the names of those candidates who the Member voting desires to support. The Scrutineers shall reject any ballot paper which supports candidates in excess of the number to be elected. They shall report to the Chairman of the meeting the number of votes cast for each candidate and the Chairman, before the close of the meeting, shall announce the names of those elected. In the case of an equality of votes for any candidate, the Chairman of the meeting shall choose between them before announcing the result of the ballot.

XVIII. The Association shall meet at times and places to be decided by the Council.

At all Ordinary General Meetings ten shall form a quorum (see also Law XIX). All meetings shall be announced by circular addressed to each Member resident in the British

Isles. At all Ordinary General Meetings the order of business shall be decided by the Chairman.

An Annual General Meeting shall, unless otherwise decided by the Council, be held on the date of the Ordinary General Meeting falling nearest to the beginning of the year.

At this Annual General Meeting the order of business shall be:

1. The reading of the minutes of the previous meeting.
2. The reading of a report of the Council on the work of the past year.
3. The statement of the Treasurer.
4. The election of Members.
5. The election of Officers and other members of the Council.
6. Other business.

A Special General Meeting may be called to discuss or take action upon any matter affecting the interests of the Association.

A Special General Meeting shall be called either by the decision of the Council or at the request of at least ten Members addressed to the Secretaries.

XIX. No new laws shall be passed nor any standing law altered or added to, nor any other change in the constitution of the Association made except by a Special General Meeting of which for this purpose fourteen days' notice must be sent to all Members resident in the British Isles.

The requisition for such a Special General Meeting duly signed and stating in writing the laws proposed or the alteration desired, must be delivered to one of the Secretaries, who shall within a reasonable period call such a meeting. The proposed new laws or alterations in the laws shall be printed in the circular convening the meeting.

At a Special General Meeting convened for the purpose of altering the constitution or amending the laws, fifteen shall form a quorum and no motion can be passed except by a two-thirds majority of those present and voting.

1 *January* 1949

Note

The following resolutions have been passed by the Council of the Association:

(1) That manuscripts reporting investigations dealing primarily with proprietary substances of which the composition or nature is not specified in such a way that the investigations can be repeated by other workers be not eligible for publication in the *Annals of Applied Biology*.

(2) That the mention of proprietary substances without specification of nature or composition be permissible in a manuscript provided that the particular substances are well known or standard and are only incidental in the work or contributory or subsidiary to the main theme or are used for purposes of control or comparative experiment.